BIODEGRADABLE CATIONIC LIPIDS IN PLASMID DNA DELIVERY

By

FUXING TANG

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Dedicated to my parents, Xianqiong Wang and Bogu Tang, and my wife, Wei Wang

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KEY TO ABBREVIATIONS

BGTC: bis-guanidinium-tris(2-aminoethyl)amine-cholesterol

Calcein-AM: Acetoxymethyl ester of calcein

diC_{14}-amidine: N-t-butyl-N'-tetradecylaminopropionamidine

CHDTAEA: cholesteryl hemidithiodiglycolyl tris(amino ethyl) amine conjugate

CHSTAEA: cholesteryl hemisuccinyl tris(amino ethyl) amine conjugate

CTAB: Cetyltrimethylammonium bromide

DC-Chol: 3-β-[N-(N', N'-dimethylaminoethanol) carbarmoyl] cholesterol

DMRIE: 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide

DOGS: dioctadecylamidoglycylspermine

DOGSDSO: 1', 2'-dioleoyl-sn-glycero-3'-succinyl-2-hydroxyetheyl disulfide ornithine conjugate

DOGSHDO: 1', 2'-dioleyl-sn-glycero-3'-succinyl-1,6-hexanediol ornithine conjugate

DOPC: 1, 2-dioleoyl-sn-glycero-3-phosphocholine

DOPE: 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine

DORIE: 1,2-dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide

DOSPA: N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride

DOTAP: 1, 2-dioleoyloxy-3-(trimethylammonio) propane

DOTMA: 1, 2-dioleoyloxypropyl-trimethylammonium chloride

DTT: Dithiothreitol

GAP-DLRIE: N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide

LHON: 6-Lauroxyhexyl ornithinate

OLON: Oleoyl ornithinate

QS3: N', N', N'-trimethylaminoethyl-1'-cholesteryl-3, 3'-dithiodipropionamide

QN3: N', N', N'-trimethylaminoethyl-1'-cholesteryl-3, 3'-dithiodipropionamide

Rh-PE: N-(lissamine rhodamine B sulfonyl)-phosphatidylethenolamine

RLU: Relative light unit

Abstract of Dissertation Presented to the Graduate School
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Fuxing Tang

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Successful gene therapy depends on efficient gene transfer vectors. Viral vectors and non-viral vectors are widely investigated. Cationic lipids are a type of promising non-viral vector, which display little immunogenicity and no potential for viral infection. It is generally believed that DNA/liposome complexes enter cells via endocytosis although other pathways such as membrane fusion may exist. The barriers involved in the transfection process *in vitro* generally include the following aspects: (1) Formation of the liposome/DNA complexes; (2) Entry of complexes into cell lines; (3) Escape of DNA from the endosomes; (4) Dissociation of DNA from liposomes; (5) Entry of DNA into nucleus; and (6) DNA transcription. The strategy to overcome any of the above barriers should increase transgene expression, and the formulations, which overcome the major barriers, will result in greater transgene expression. Toxicity and low transfection efficiency compared to viral systems are two major barriers limiting the clinical

applications of cationic lipids. Hundreds of cationic lipids have been synthesized to address these problems. The focus of this dissertation were designed to use biodegradable cationic lipids to enhance transgene expression and decrease toxicity of cationic lipid-mediated plasmid DNA delivery. Biodegradable disulfide cationic lipids-1', 2'-dioleoyl-sn-glycero-3'-succinyl-2-hydroxyethyl disulfide ornithine conjugate (DOGSDSO) and cholesteryl hemidithiodiglycolyl tris(aminoethyl)amine (CHDTAEA), and a single tailed-cationic lipid-containing an ester bond in the backbone-6-lauroxyhexyl ornithinate (LHON) were designed, synthesized, and investigated for plasmid DNA delivery. The helper function of 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was investigated. The results demonstrated that liposomes made of DOGSDSO/1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and CHDTAEA/DOPE could be degraded by cell lysate or glutathione. DOGSDSO or CHDTAEA liposomes had greater transfection activity than its non-disulfide analogs. CHDTAEA liposomes also resulted in less toxicity than its non-disulfide analog. Single-tailed LHON had greater transfection acitvity than double-tailed 1, 2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP). Introduction of an ester bond to the backbone of LHON decreased cytotoxicity. DOPC demonstrated superior helper function in certain formulations in CHO cells. The results demonstrated that use of biodegradable cationic lipids for plasmid DNA delivery could decrease cytotoxicity and increase the transgene expression. DOPC could function as a helper lipid in certain formulations or cell lines.

CHAPTER 1
INTRODUCTION

Background

Gene therapy provides a paradigm for the treatment of human diseases (Anderson 1992, Anderson 1998, Crystal 1995, Miller 1992, Mulligan 1993). The ultimate goal of gene therapy is to cure both inherited and acquired disorders by attacking the original causes, i.e. adding, blocking, correcting, or replacing genes. Although gene therapy trials have been initiated worldwide for more than two decades, successful therapeutic outcomes have been limited. One of the major hurdles for successful gene therapy is the lack of an efficient gene delivery system. An ideal gene delivery system should be specifically targeting, biodegradable, non-toxic, non-immunogenic, and stable for long term storage. Vectors for gene delivery have been categorized into viral and nonviral.

Viral vectors are created to transfer therapeutic genes into patients by genetic engineering. To avoid causing viral diseases, only attenuated or defective viruses are used (Anderson 1992, Fisher et al. 1999). Taking advantage of the ability to infect cells, several viruses, notably retrovirus, adenovirus, and adeno-associated virus have been widely investigated in gene delivery *in vivo*.

Retrovirses were the first viral vectors used in gene therapy (Miller 1992). Retroviruses are RNA viruses, which have the ability to insert their genes permanently into the host cell chromosomes (Varmus 1988). The advantages of retroviral vectors include: high transduction efficiency, therapeutic gene insertion size up to 8 kb, and

vector proteins are not expressed in host. The disadvantages are that: retroviral vectors only infect dividing cells, random integration may cause fatal mutagenesis, only low titer number can be achieved (10^7-10^8), *in vivo* delivery efficiency is low (usually needs to infect helper cell lines *in vitro* then cells are administered *in vivo*).

Adenoviruses are DNA viruses that can infect both dividing and non-dividing cells (Smith et al. 1993). Unlike retroviruses, adenoviruses do not integrate into the chromosomes of the host cells. Genes introduced into cells using adenoviral vectors are maintained in the nucleus of host cells as an extrachromosomal element and only can provide expression for a limited period of time. The advantages of adenoviral vectors are that the vectors can infect nearly all cell types, insertion size up to 7.5 kb, and high titers up to 10^{12} titers. The disadvantages are transient expression, humoral immune response, and the possibility to further express viral proteins.

Adeno-associated virus (AAV) is a single-stranded DNA virus capable of permanently inserting their genome into the chromosomes of the host cells (Kotin 1994, Muzyczka 1992). AAV can infect both dividing and non-dividing cells. These vectors have the potential to be designed to be site-specific integration and nonpathogenic. The major limitation is that AAV cannot incorporate therapeutic genes larger than 5 kb.

The limitations of viral systems make synthetic vectors an attractive alternative for gene therapy (Felgner et al. 1994, Felgner et al. 1987, Gao and Huang 1996, Hickman et al. 1994, Wolff et al. 1990, Yang et al. 1990, Zhou et al. 1991). Plasmid-based approaches of gene therapy are often termed as non-viral methods. Several advantages of non-viral vectors includes: non-immunogenicity, low acute toxicity, simplicity, and feasibility to be produced on a large scale. Limitations of non-viral vectors include their

transient gene expression and lower efficiency than viral vectors. Non-viral approaches can be categorized into naked DNA, gene gun, liposome/DNA complexes, polymer/DNA complexes, and liposome/polymer/DNA complexes.

Naked DNA has been used in gene transfer to skeletal muscle (Wolff et al. 1990), liver (Hickman et al. 1994, Liu et al. 1999a), heart muscle (Ardehali et al. 1995), and tumor (Yang and Huang 1996). However, free DNA needs to be protected from degradation by nucleases when systemically administered (Liu et al. 1997).

When the target tissues are surgically exposed, DNA can be injected into tissues using a bioballistic bombardment or gene gun (Yang and Huang 1996, Sun et al. 1995). The method is simple, efficient and applicable for dermal delivery. However, the applications are limited by the requisite of the exposure of tissues.

Cationic liposomes interact with DNA to form liposome/DNA complexes (Lipoplex) (Felgner et al. 1987, Gao and Huang 1991). The positive charges of liposomes or formation of the complexes can protect DNA from degradation by nucleases, and also help the lipoplex to attach to the cell surface so as to initiate endocytosis or membrane fusion process (Gao and Huang 1995).

Cationic polymers can also complex with DNA and transfer DNA *in vitro* and *in vivo* (Gao and Huang 1996, Zhou et al. 1991). Several polymers such as polylysine, polyethylenimine, and dendrimers have been used in gene delivery (Pollard et al. 1998). Huang's group incorporated polylysine into cationic liposomes/DNA or anionic liposomes/DNA complexes to form-liposome-entrapped, polycation-condensed DNA (LPDS)-LPDI and LPD II (Gao and Huang 1996, Li and Huang 1997). The formation of

liposome/polymer/DNA complexes can reduce the size of complexes and increase gene delivery efficiency (Gao and Huang 1996, Li and Huang 1997).

Cationic liposomes are the most extensively investigated non-viral vectors. 3-β-[N-(N', N'-dimethylaminoethanol) carbarmoyl] cholesterol (DC-Chol), 1, 2-diolyoxy-3-(trimethylammonio) propane (DOTAP), and 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE) were used in clinical trials to treat cystic fibrosis and cancer (Hyde et al. 1993, Nabel et al. 1993, Parker et al. 1995). Cationic liposomes are usually composed of a cationic lipid and most of time a neutral helper lipid such as 1, 2-dioleoyl phosphatidylethanolamine (DOPE) or cholesterol to increase gene delivery efficiency *in vitro* or *in vivo*.

Problems and Reseach Projects

Potential Mechanism of Cationic Lipid-Mediated Plasmid Delivery

It is generally believed that DNA/liposome complexes enter cells via endocytosis although other pathways such as membrane fusion may exist (Gao and Huang 1995, Rolland 1998). The barriers involved in the transfection process *in vitro* generally include the following aspects (Zabner et al. 1995): 1. Formation of the liposome/DNA complexes; 2. Entry of complexes into cell; 3. Escape of DNA from the endosomes; 4. Dissociation of DNA from liposomes; 5. Entry of DNA into nucleus; and 6. DNA transcription. The strategy to overcome any of the above barriers should increase transgene expression, and the formulations, which overcome the major barriers, will result in greater transgene expression (Figure 1-1).

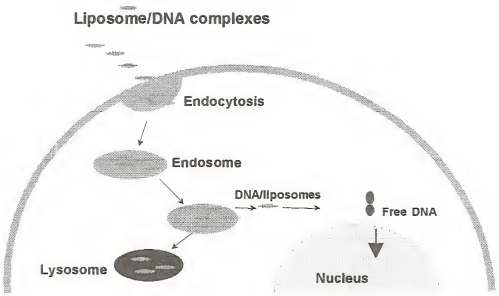


Figure 1-1: Potential mechanism of cationic lipid-mediated plasmid DNA delivery.

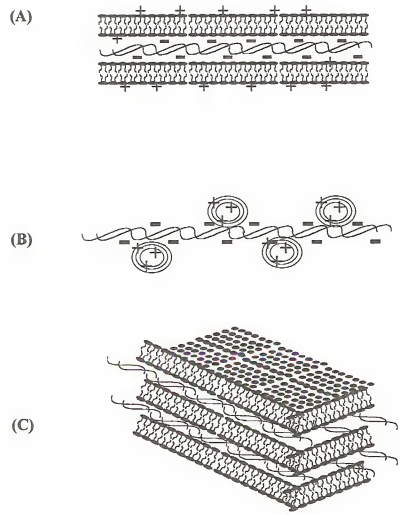


Figure 1-2: Cross-fraction structures of DNA/liposome complexes. (A) Spaghetti structure. The positively charged liposomes coat DNA strand with a cylindrical-shaped bilayer. (B) Beads on a string structure. Positively charged liposomes adhere as an intact bead on the negatively charged DNA strand. (C) Multilamellar structure. DNA strand is intercalated in mulilamellar liposome membrane.

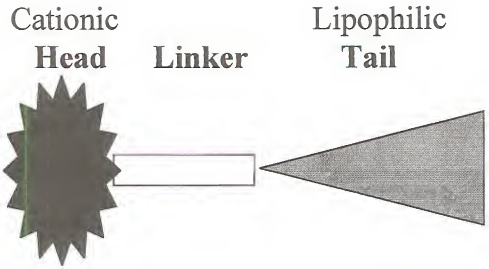


Figure 1-3: Basic structure of cationic lipids.

The Structure of DNA/liposome Complexes

DNA/liposome complexes are heterogeneous with respect to size, shape, and composition. Three types of structures have been reported (Figure 1-2). The traditional application of liposomes is to deliver drug via encapsulating the compound in the aqueous or bilayer lipid part of liposomes. However, there is no true encapsulation of DNA by the cationic liposomes since cationic liposomes are fused and a large part of the aqueous content is released from liposomes when liposomes fuse with each other upon DNA interacting with liposmes (Noguchi et al. 1998, Wasan et al. 1998). Using freeze-fracture electron microscopy, Huang and coworkers identified that DNA/DC-Chol/DOPE complexes were mainly composed of tubular spaghetti-like structures (Figure 1-1A), or a combination of tubular spaghetti-like structures and spaghetti-meatball assemblies (Figure 1-2A and Figure 1-2B) (Sternberg et al. 1994). Using optical and X-ray diffraction, Safinya and coworkers reported the complexes consisted of a high ordered multilamellar structure with DNA sandwiched between the cationic lipid bilayers (Figure 1-2C) (Koltover et al. 1998, Radler et al. 1997). Among the three representative structures, mulitilamellar structure (Figure 1-2C) was calculated to be the most stable using a model for lipid self-assembly (Dan 1998). The relationship between complex structure and transgene expression activity is still unknown since it is difficult to separate a complex of particular structure from the heterogeneous population of complexes. The structures of complexes are dependent on composition of cationic lipid and the presence of neutral helper lipid. The commonly used helper lipid DOPE induces the lamellar (L^C_α) $\rightarrow$ two-dimensional hexagonal lattice (H^C_{II}) structural transition by controlling the spontaneous curvature of the lipid monolayer. In contrast, 1, 2-dioleoyl-sn-glycero-3-

phosphocholine (DOPC) does not cause the structural change (Koltover et al. 1998). They also reported that H^{C}_{II} instead of L^{C}_{α} complexes fuse and release DNA upon contact with anionic vesicle (Koltover et al. 1998). These findings provided a rationale to explain why DOPE is a superior helper lipid compared to DOPC in many cationic liposome-mediated plasimd delivery systems (Farhood et al. 1995, Felgner et al. 1994).

The Limitations of Cationic Lipid-mediated Plasmid DNA Delivery

Two major problems have limited the applications of cationic lipids in clinical trial; one is toxicity, another is low efficiency compared to viral system. Over the last decade, hundreds of cationic lipids have been synthesized to overcome these two limitations. However, the synthesis strategy is still based on so-called random design since it is very hard to predict the transfection activity of a cationic lipid from the chemical structure (Lee et al. 1996). The inability to predict transfection activity of cationic lipids may be due to lack of understanding the mechanism of cationic lipid-mediated plasmid DNA delivery, the cellular variability, the lack of relationship between *in vitro* and *in vivo* experiments, and difference of the intracellular distribution and trafficking of complexes among different types of cells.

The Structure of Cationic Lipids

A cationic lipid is usually composed of three parts, a cationic head group, a lipophilic tail group, and a linker which tethers the hydrophilic head group and hydrophobic tail group (Figure 1-3). The tail groups are usually composed of saturated or unsaturated alkyl or cholesteryl group(s). Felgner and coworkers reported that the transfection activity of cationic lipids decreased with increasing of length of saturated aliphatic chain from 14 to 16 or 18 carbon atoms (Felgner et al. 1994). Increasing the

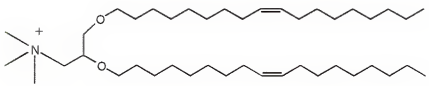
aliphatic chain length of cationic lipid increases the phase transition temperature and stiffness of liposomes thus affecting the transfection efficiency. Since bilayer rigidity can be decreased by introducing unsaturated into alkyl chain(s), unsaturated chain(s) are preferred to saturated in long chain tail(s) (Felgner et al. 1994). Although hundreds cationic lipids were synthesized to improve transgene expression and decrease toxicity, cationic lipids can basically be categorized into four different groups according to the type of head groups: 1. Quaternary ammonium salt lipids, 2. Lipoamines (primary, secondary, and tertiary amines lipids), 3. Cationic lipids containing both lipoamine and quaternary amine in the head groups, and 4. Amidinium salt lipids and their miscellaneous cationic lipids (Table 1).

Quaternary ammonium salt lipids

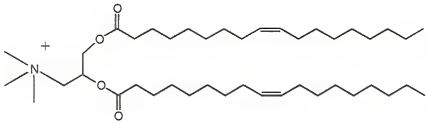
1, 2-dioleoyloxypropyl-trimethylammonium chloride (DOTMA) (Figure 1-4) was the first lipid used for plasmid DNA delivery (Felgner et al. 1987). This lipid was proposed to use alone or in combination with a neutral additive lipid (helper lipid) such as DOPC or DOPE to form liposomes. The ether bond in DOTMA is not biodegradable and thus results in intracellular accumulation of toxic lipids. In order to decrease the cytotoxicity, di-ester bonds were introduced in DOTAP to replace di-ether in DOTMA, resulting in less toxicity. The quaternary ammonium lipids of this family were modified by replacing the methyl group with an alkylene alcohol or the alkylene tail group with an alkyl group to give 1,2-dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DORIE) or 1,2-dimyristyloxypropyl-3-dimethyl- hydroxyethyl ammonium bromide (DMRIE) (Figure 1-4) (Felgner et al. 1994). The hydroxyl group in DORIE or DMRIE

Table 1-1: Different head groups, linkers, and tails of cationic lipids and examples.

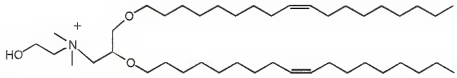
Head group	**Linker**	**Tail group**
Quaternary ammonium (DOTMA)	Ether (DOTMA)	Alkyl (DMRIE)
Lipoamine (DC-Chol)	Ester (DOTAP)	Alkenyl (DOTAP)
Quaternary +Lipoamine (DOSPA)	Carbamate (DC-Chol)	Cholesteryl (DC-Chol)
Amidinium (diC_{14}-amidine)	Disulfide (DOGSDOS)	
Miscellaneous (BGTC)		



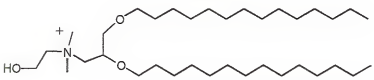
DOTMA



DOTAP



DORIE



DMRIE

Figure 1-4: Structures of DOTMA, DOTAP, DORIE, and DMRIE

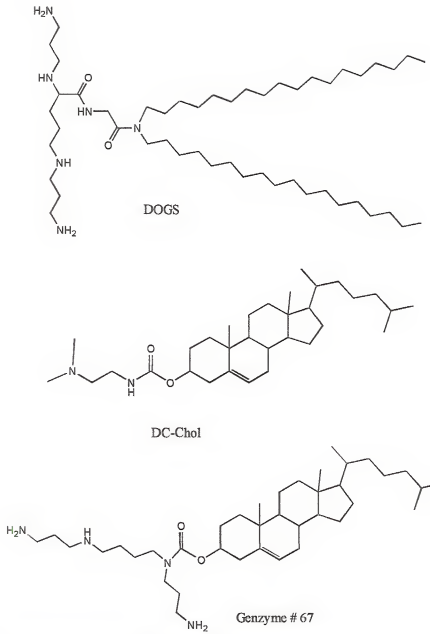
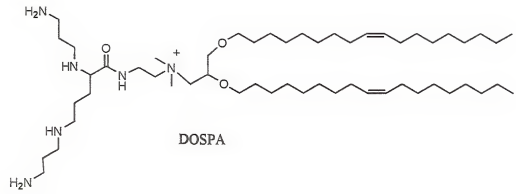
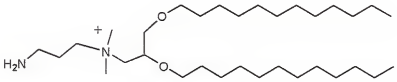


Figure 1-5: Structures of DOGS, DC-Chol, and Genzyme # 67.



DOSPA



GAP-DLRIE

Figure 1-6: Structures of DOSPA and GAP-DLRIE.

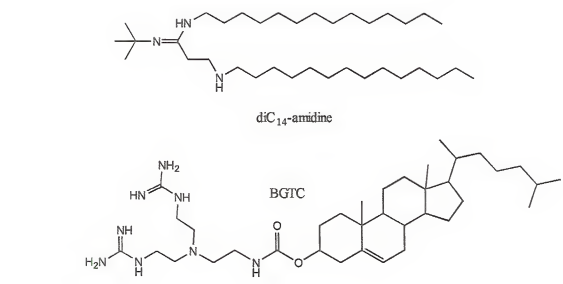


Figure 1-7: Structures of diC_{14}-amidine and BGTC.

may increase the interaction of DNA with lipids or improve the interaction of the DNA/liposome complexes with cellular membranes, leading to greater activity compared to DOTMA or DOTAP *in vitro* and *in vivo* (Felgner et al. 1994, San et al. 1993, Wheeler et al. 1996).

Lipoamines

An alternative approach of using quaternary amine lipids was first proposed by Behr and coworkers (Behr et al. 1989). Taking advantage of a naturally nucleus-occurring polyamine which plays a role in DNA compaction during cell division, Behr synthesized the cationic lipid-dioctadylamidoglycylspermine (DOGS) (Figure 1-5) for plasmid delivery. DOGS does not need to be formulated with a neutral lipid to achieve a high level of transgene expression *in vitro* and *in vivo* (Remy et al. 1994). Tertiary amines result in less protein kinase inhibition than quaternary amines, thus potentially reducing side effects (Farhood et al. 1992). To avoid the side effects of the quaternary amine, Huang and Gao introduced DC-Chol lipid (Figure 1-5). DC-Chol lipid contains a tertiary amine head group instead of a quaternary amine group. The low toxicity of DC-Chol led it to be the first cationic lipid applied for clinical trials (Caplen et al. 1995a). Lee and coworkers from Genzyme Corporation extended Huang and Gao's idea and synthesized a series of derivatives of cholesteryl carbamate cationic lipids (Lee et al. 1996). The cationic lipid #67 demonstrated the greatest transfection activity *in vivo* among the tested derivatives (Figure 1-5). Lee showed that polyamine lipid #67 had greater transfection activity than monoamine DC-Chol *in vivo*. However, the further increase in protonable amine groups of #67 from 3 to 4 resulted in a decrease in transfection activity (Lee et al. 1996). These results suggested that other factors such as

the balance between head group and tail group, in addition to the number of amines in the head group, were also important for optimal transfection activity.

Cationic lipids containing both lipoamine and quaternary amine in the head groups

Lipoamines have buffering functions, which inhibits the early endosomes to mature and delays the fusion between endosomes and lysomes, thus increasing the half-life of plasmid DNA in cells. The buffering property of lipoamines was rationalized to increase transfection activity (Legendre and Szoka 1992). Quaternary amines do not have buffering function since they are always charged at any pH. However, quaternary ammonium cationic lipids such as DOTAP have stronger interactions with DNA than lipoamines such as DC-Chol (Zuidam and Barenholz 1998). A combination of two types of head groups was proposed to increase transfection efficiency (Wheeler et al. 1996). The first cationic lipid containing quaternary amine and lipoamines in the head group is N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride (DOSPA) from Vical Corporation (Figure 1-6). When formulated with the helper lipid-DOPE to form liposome-"Lipofectamine", DOSPA indeed had a high level of transfection activity (Wheeler et al. 1996). However, its toxicity prevented it from further clinical development. N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE) (Figure 1-6) contains one primary amine and a quaternary amine and a shorter dodecyl tail groups (Wheeler et al. 1996). GAP-DLRIE demonstrated low toxicity and greater transfection activity than DOPSPA and DOTMA *in vitro* and *in vivo* (Wheeler et al. 1996). However, no rationale was given to explain the decrease of toxicity.

Amidinium salt lipids and miscellaneous cationic lipids

Ruysschaert and coworkers first reported the application of amidine as a head group in the cationic lipid-N-t-butyl-N'-tetradecyl-3-tetradecylaminopropionamidine (diC_{14}-amidine) (Ruysschaert et al. 1994) (Figure 1-7). Vesicles made of diC_{14}-amidine and DOPE or pure diC_{14}-amidine were efficient in the delivery of plasmid DNA to adherent Chinese hamster ovary (CHO) and suspension K562 cell lines. Lehn's group combined bis-guanidinium group with cholesterol to give bis-guanidinium-tris(2-aminoethyl)amine-cholesterol (BGTC) (Figure 1-7) (Oudrhiri et al. 1997, Vigneron et al. 1996). One advantage of BGTC is that it can be used as a colloidal solution, therefore, avoiding the inconvenience of liposomes formulation, such as consistency in size, stability while transportation (Vigneron et al. 1996). *In vivo* data demonstrated that BGTC could transfer plasmid DNA to mouse airway epithelial cells (Oudrhiri et al. 1997).

Cationic Lipids Containing Disulfide Linker

Toxicity is one of the major barriers that limits the application of cationic lipids in clinical trials. The use of ester, amide, and carbamate linkages to tether cationic head group and hydrophobic domains is the common strategy to lower toxicity. Ester bonds are biodegradable, however, the introduction of the ester bond may also decrease the stability of liposomes in the systemic circulation. For example, Aberle and coworkers constructed a tetraester cationic lipid and the liposome demonstrated lower toxicity in NIH 3T3 than DC-Chol. However, this liposome must be used within 2 hours of preparation (Aberle et al. 1998). Carbamate is biodegradable and more stable than an ester bond in aqueous solutions. Huang's group first introduced the carbamate into a

cationic lipid DC-Chol (Gao and Huang 1991). DC-Chol was the first lipid used in clinical trials because of its combined properties of transfection efficiency, stability, and low toxicity (Gao and Huang 1995). Another barrier for cationic lipid-mediated plasmid DNA delivery is the low transfection efficiency compared to the viral system. The projects in the current work have been designed to decrease toxicity and increase transfection activity of cationic lipids.

One of the strategies is to take advantage of the high intracellular reductive environment to use a disulfide linker-containing cationic lipid, which is stable outside cells whereas it can be reduced in cells by intracellular reductive substances such as glutathione. The reduction of a disulfide linker is hypothesized to result in the collapse of liposomes thereby, enhancing the release of DNA from DNA/liposomes complexes. Dissociation of DNA from DNA/liposomes complexes is one of major barriers for cationic liposome-mediated gene transfection (Escriou et al. 1998a, Rolland 1998, Zabner et al. 1995). The enhancement of the released DNA is expected to increase transgene expression. The biodegradability of disulfide cationic lipids would decrease toxicity.

Biodegradable Single-tailed Cationic Lipid

Two major types of hydrophobic moieties are used in cationic lipids; one is a pair of aliphatic chains, and the other is based on cholesterol. Huang and coworkers reported single-tailed cationic lipids, such as cetyl trimethylammonium bromide (CTAB), to be more toxic and less efficient than their double-tailed counterparts, such as N- [1-(2,3-dioleyloxy) propyl]-N,N,N-tri-methylammonium chloride (DOTMA) (Pinnaduwage et al. 1989). According to published results, neither the head group nor the tail(s) of the lipid are the determinant for cationic lipids being used as plasmid delivery vectors (Gao and

Huang 1995, Rolland 1998). Therefore, it was hypothesized that with a suitable head group, single-tailed cationic lipids may function in gene delivery and introducing an ester bond in the tail backbone may decrease the toxicity of single tailed-lipid. To test these hypotheses, one project is to design and synthesize a biodegradable single-tailed cationic lipid and investigate its transfection.

Novel Helper Function of 1, 2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC)

Most cationic liposomes containing a helper lipid-1, 2-dioleoyl phosphoethanolamine (DOPE) work more efficiently than formulations without DOPE *in vitro* (Farhood et al. 1995, Felgner et al. 1995, Gao and Huang 1991, Lee et al. 1996, Tang and Hughes 1998, Xu and Szoka 1996, Yang and Huang 1998). The replacement of DOPE with another neutral lipid-1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), was reported to reduce transfection activities of cationic liposomes (Farhood et al. 1992, Farhood et al. 1995, Felgner et al. 1994, Legendre and Szoka 1992, Zhou and Huang 1994). In preliminary experiments, an unexpected finding was that liposomes containing DOPC demonstrated greater transfection activities in CHO cells compared to those containing DOPE in some liposome formulations. We hypothesized that DOPC may function as a superior helper lipid than DOPE in certain cells lines or in certain liposome formulations. The third part of the project is to investigate the helper function of DOPC lipid in some formulations or in some cell types and try to obtain insight of mechanisms of cationic lipid-mediated gene transfection.

CHAPTER 2
INTRODUCTION OF A DISULFIDE BOND INTO A CATIONIC LIPID INCREASES TRANSGENE EXPRESSION OF PLASMID DNA

Introduction

Cationic liposomes are popular non-viral gene transfer vectors. Significant efforts have been made to improve these vectors in many academic and industrial laboratories. Research has focused on the synthesis of new cationic lipids, the development of better formulations, and improvements in plasmid design (Felgner et al. 1994, Gao and Huang 1991, Liu et al. 1996). Although the disulfide linkage has been used in drug delivery with promising results (Trail et al. 1997, Trail et al. 1993), the introduction of a disulfide linkage into cationic lipids has not been fully investigated. In this chapter, we described the synthesis of a cationic lipid, 1', 2'-dioleoyl-sn-glycero-3'-succinyl-2-hydroxyethyl disulfide ornithine conjugate (DOGSDSO) (Figure 2-1). The working hypothesis for the current studies is that after the DOGSDSO condensed plasmid DNA particle is endocytosed, the reductive substances that reside in the cell (i.e. endosome, cytosol, etc) will degrade the disulfide bond. The breaking of this bond will result in the collapse of liposome/DNA complexes, leading to the dissociation of DNA from the cationic lipid. This specific characteristic of disulfide linkage might increase transgene expression, as the dissociation of DNA from the cationic liposome is one of the major barriers of transgene expression (Zabner et al. 1995). We reported that dithiothreitol (DDT) and cell lysate could destabilize disulfide cationic liposome/DNA complexes and

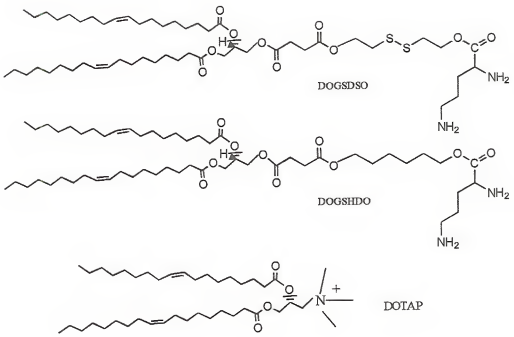


Figure 2-1: Chemical structures of DOGSDSO, DOGSHDO and DOTAP.

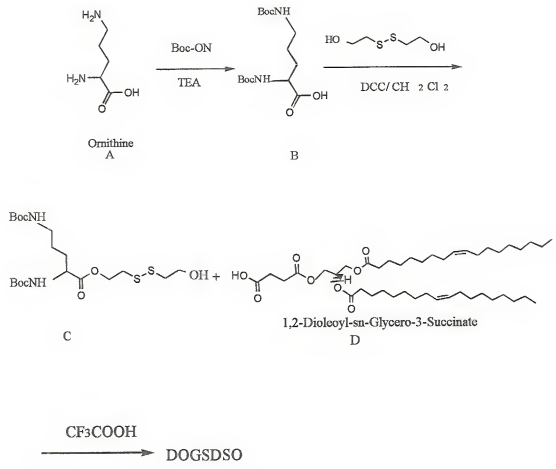


Figure 2-2: Schemes for the synthesis of DOGSDSO. Boc-ON, 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile; TEA, Triethylamine; DCC, 1,3-Dicyclohexylcarbodiimide.

release DNA. In addition, we have ensured the alterations in gene expression were not from a generalized increase in cellular associated plasmid DNA. DOTAP liposomes were included as a positive control in all experiments since it has been used in many *in vitro* and *in vivo* experiments (Gao and Huang 1995, Rolland 1998) and serves as good benchmark to evaluate new cationic lipids (Figure 1-1). Overall, the results of these studies demonstrate that cationic lipids containing a disulfide linkage may be a promising gene delivery vector.

Materials and Methods

Synthesis of DOGSDSO (Figure 2-2)

Amino groups of L-ornithine were protected with Boc-ON in wet 1,4-dioxane to produce B. B was esterified with 5 equivalent of 2-hydroxyl disulfide to form C (DCC, 1.1 equiv., RT , in CH_2Cl_2). C and D (1:1) were condensed to generate Boc-DOGSDSO (DCC, 1.1 equiv., RT, in CH_2Cl_2). All reactions were followed by TLC. A normal separation method of silica gel chromatography was used to purify B, C, D and E. The molecular weight of E was determined by ionization spray positive mode mass spectrometry, M=1171(calculated 1171.7 for $C_{62}H_{110}N_2O_{14}S_2$). ^{1}H NMR($CDCl_3$, 300 HZ): 0.90 [t, 2X(CH_3)], 1.20-1.69[m, 26X(CH_2)], 1.44[s, 2X(CH_3)$_3$], 2.01 [q, 4X(C=C-CH_2)], 2.31 [m, 2X(CH_2C=O)], 2.71 [s, O=CCH_2CH_2C=O], 2.91 [t, CH_2SSCH_2], 3.12 [q, CH_2N], 4.18 [m, a-H], 4.36 [2X(CH=CH)], 4.62 [broad s, NH], 5.05-5.12[broad s, NH], 5.22-5.40 [m, OCH_2CHOCH_2O]. Before used to prepare liposomes, E was deprotected (CF_3COOH, 0^0C, 15min) (Behr et al. 1989) and excessive TFA was dried under high vacuum to yield DOGSDSO. The overall yield was 55%-60% based on the amount of amino acid. Because DOGSDSO was unstable on silica gel while

chromatography, we checked its purity by TLC and it was used directly to prepare liposome. We also used same conditions to synthesize its analog DOGSHDO.

Chemicals

DOTAP, DOPE, and 1,2-dioleoyl-sn-glycero-3-succinate were purchased from Avanti Polar Lipids (Alabaster, AL). Acetoxymethyl ester of calcein (calcein-AM) was purchased from Molecular Probes, Inc. (Eugene, OR). Other chemicals were purchased from Aldrich (Milwaukee, WI).

Plasmid DNA

All supplies were purchased from Promega (Madison, WI). Luciferase and green fluorescence protein (GFP) plasmids were obtained from E. coli (strain JM-109) which had been transfected with a pGL-control (with SV40 promoter) luciferase producing plasmid or a pUF2 (with CMV promoter) plasmid (a kind gift from N. Muzyczka, University of Florida) (Klein et al. 1998). Plasmid DNA was isolated using a Wizard DNA Purification kit (Promega). The concentration and purity of pDNA were determined by absorbance at 260 nm and 280 nm.

Liposome Preparation

DOTAP, DOGSDSO, and DOGSHDO were dissolved and mixed with a neutral lipid DOPE (1:1 molar ratio) in chloroform respectively. The mixture was evaporated to dryness in a round-bottom flask using a rotary evaporator at room temperature. The lipid film was dried by nitrogen for further ten minutes to make sure all solvent evaporated. The lipid film was suspended in sterile water to make a concentration of 1 mg/ml based the amount of DOTAP, DOGSDSO or DOGSHDO. The resultant mixtures were shaken for 30 minutes, followed by sonication for 5 minutes to form homogenized liposomes.

The particle size distribution of the liposomes and zeta potential were measured by using a NICOMP 380 ZLS instrument (Santa Barbara, CA). The volume-weight distribution parameters of DOTAP, DOGSDSO, and DOGSHDO liposomes were 96±24 nm, 58±12 nm, and 57±12 nm respectively. The zeta potentials of DOTAP, DOGSDSO and DOGSHDO liposomes were +25.32 mV, +12.78 mV, and +11.18 mV, respectively. The liposomes were stored at 4 ^{0}C until use.

Dissociation of DNA from Liposome/DNA Complexes in Reductive Media

DNA was complexed with DOGSDSO and DOGSHDO liposomes respectively. After complexes were incubated for 30 minutes at room temperature, a solution of DL-dithiothreitol (DTT) in PBS (pH=7.4) was added to complexes to make a DTT concentration of 10 mM. The mixtures were incubated for 12 hours at 37 ^{0}C . Free DNA was visualized by SYBR staining (1XSYBR in 0.7% agarose gel, 75 V, 2 hours).

Preparation of Cell Lysate

One $\times 10^7$ Chinese hamster ovary (CHO) cells were collected and centrifuged. The cell culture media was decanted and cells were suspended in 0.5 ml of sterile water. The cell suspension was transferred to a 1.5 ml of test tube. The cell membrane was ruptured by three cycles of freezing & thawing (acetone-dry ice solution and 37 ^{0}C-water bath). The suspension was centrifuged at 10,000 × g at 4 ^{0}C. The supernatant was transferred and stored at – 80 ^{0}C until use.

Release and Digestion of Plasmid DNA from DNA/cationic Liposome Complexes by Cell Lysate

Ten micrograms of plasmid (pGL3) was complexed with 80 micrograms (calculation based on the weight cationic lipid) of DOGSDSO/DOPE and DOGSHDO/DOPE in test tubes, respectively. The mixtures were incubated at room

temperature for 30 minutes. The DNA/liposome complexes were mixed with 200 µl of cell lysate or 200 µl of sterile water and followed by an incubation of 6 hours at 37 ^{0}C. After the incubation period, the mixtures were quenched with 100 µl of saturated phenol water solution. 300 µl of chloroform was added to each test tube to extract phenol and cationic lipids. The mixtures were centrifuged at 5000 x g for 1 minute and 20 µl of the upper aqueous layers of each treatment was loaded on a 0.7 % agarose gel for electrophoresis.

Flow Cytometry

To analyze cellular associated DNA delivered by liposomes, SKnSH cells were plated at 7×10^{5} cells/well and fluorescein-labeled plasmid DNA was delivered by DOGSDSO/DOPE and DOGSHDO/DOPE with a ratio of cationic lipid to DNA of 4:1 and 8:1, respectively. Fluorescent labeled plasmid DNA was prepared by using a Fluorescein Labeling Kit from Mirus (Madison, WI). After a forty-eight hours post-transfection, the cells were washed and lifted from the plate with 200 µl of trypsin-EDTA, and then transferred into Eppendorf tubes and centrifuged at 150 X g for 5 minutes. The cells were washed twice, resuspended in 800 µl of PBS and kept on ice until analysis. Cell fluorescence was monitored using a Becton Dickinson FACSort flow cytometer (San Jose, CA). 10,000 cells were counted for each treatment. Cells were gated with their morphological properties, forward scatter and side scatter set on a logarithmic scale. The mean fluorescence intensity of the particular population of cells was measured in terms of arbitrary units using a LYSYS II software program (Becton Dickinson, San Jose, CA). Each experiment was performed in triplicate.

Cell Culture and Luciferase Gene Transfection

COS 1 and CHO cells were obtained from American Tissue Type Collection (Rockville, MD) and maintained in D-MEM (4.5g/ml glucose) and α-MEM, respectively, and supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml). All cells were maintained in 5% CO_2 and at 37 ^{0}C in humidified air. Cell lines were cultured and seeded in 24 well plates (2×10^5 cells/well) and grown to 60-80 % confluence in 1 ml of serum containing-media. DNA/lipid complexes were made and incubated at room temperature in serum free media for 5-10 minutes before being used for transfection. Prior to transfection, growth media containing serum was changed to serum free media. In order to test serum sensitivity, the growth media were changed to fresh growth media and the transfection mixtures added to the cells. After a pre-set period, the media was changed to growth media containing serum, and the cells were grown for another 48 hours. Luciferase activity was measured using a standard luciferase assay. Luciferase activity in cultured cells was expressed as RLU per mg of lysate protein. Each experiment was repeated at least twice, and experiments were done in triplicate within a given series.

GFP Gene Transfection

The optimal weight ratio for transfection of each liposome was used. Three μg per well of pUF2 DNA was complexed with 30 μg of DOGSDSO/DOPE, 30 μg of DOGSHDO/DOPE, and 6 μg of DOTAP/DOPE (calculation based on cationic lipid), respectively. Complexes were incubated with CHO cells in serum free media for 4.5 hours. The media was then changed to growth media and further incubated for 20 hours. Transfection efficiency was determined by counting the number of green fluorescence

cells compared with all cells in the field of vision under 20× magnification using a Nikon Diaphot inverted microscope equipped with a GFP filter cube (Chroma Technology Corp., Japan). Each experiment was conducted in triplicate and three separate counts were made for each well.

Statistical Analysis

Statistical analyses were performed using ANOVA-Fisher's PLSD Post hoc tests (Stat View 4.53, Abacus Concepts, Inc., Berkeley, CA). One-way ANOVA was applied to compare transfection activity difference between different liposomes/DNA ratios of the same liposome. Two-way ANOVA was applied among treatments of liposome groups and liposomes/DNA ratios or overtime. A probability of less than 0.05 was considered to be statistically significant.

Results

Release of Plasmid DNA from Liposomes by DTT

To evaluate dissociation of DNA from complexes in reductive media, we treated liposome/DNA complexes either with media of 10 mM DTT or DTT free for 12 hours at 37 ^{0}C. Only DNA complexed by DOGSDSO liposome and treated with DTT was released (Figure 2-3) . DNA complexed by DOGSHDO liposome was not released at in either a reductive or non-reductive environment. If not treated by DTT, no DNA was released from DOGSDSO liposome complexed after incubated for 12 hours at 37 ^{0}C. The results implied that dissociation of DNA from liposome complexes is not spontaneous and may act as a barrier for gene transfection. Exposed to the high intracellular reductive substances, disulfide bond of DOGSDSO may be reduced and DNA may be more readily released to cytoplasm and increase transfection activity.

Release and Digestion of Plasmid DNA from DNA/cationic Liposome Complexes by Cell Lysate

Cationic liposomes can complex with plasmid DNA and protect pDNA from digestion by nuclease. In this experiment if the liposome complex is disrupted the liberated DNA would become susceptible to degradation by cellular nucleases. As shown in Figure 2-4, the complexes of non-disulfide cationic liposome DOGSHDO/DOPE/DNA were stable in both non-reductive and reductive environments (i.e. sterile water and cell lysate). The extracted amount of DNA was not significantly different from each other; thus the amount of released DNA from complexes was not significant. Complexes of disulfide cationic liposome DOGSDSO/DOPE/DNA were not stable in the cell lysate and the majority of DNA that was released form the complexes was digested. However, the disulfide liposome/DNA complexes were stable in the non-reductive environment and the extracted amount of DNA was comparable to that obtained from non-disulfide liposome complexes.

Cellular Associated pDNA Delivered by DOGSDSO/DOPE and DOGSHDO/DOPE

To test whether the enhancement of transgene expression transfected by DOGSDSO/DOPE liposomes was caused by a greater amount of pDNA delivery instead of intracellular events, we used flow cytometry to quantify cellular associated pDNA after delivery. As we initially reported that at the ratio of DNA to cationic lipid of 1: 4, DOGSDSO/DOPE had higher transfection than DOGSHDO/DOPE in SKnSH cell line (Tang and Hughes 1998). However, as indicated in Figure 2-5, cells which were treated by DNA/(DOGSDSO/DOPE) complexes had lower cellular associated FITC-pDNA than

A B C D

Figure 2-3: Electrophoretic analysis of binding of 1 μg of pDNA with 16 μg liposomes and releasing of pDNA form complexes in reductive media. A. DOGSDSO liposome/pDNA; B. DOGSDSO liposome/pDNA + 10 mM DTT; C. DOGSHDO liposome/pDNA; D: DOGSHDO liposome/pDNA +10 mM DTT.

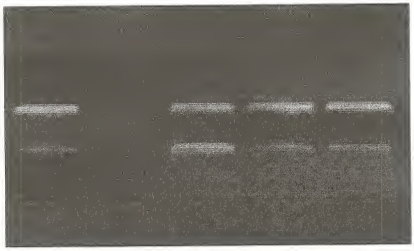


Figure 2-4: Release and digestion of pDNA by cell lysate. Ten μg of plasmid DNA was complexed with cationic liposomes DOGSDSO/DOPE and DOGSHDO/DOPE at the ratio of 1:8 (w/w) respectively. The complexes were incubated in the absence or presence of cell lysates, and the results were analyzed by agarose gel electrophoresis as described in Methods. A: Control DNA; B: DNA / (DOGSDSO/DOPE) + cell lysate; C: DNA / (DOGSDSO/DOPE); D: DNA / (DOGSHDO/DOPE) + cell lysate; E: DNA / (DOGSHDO/DOPE).

those treated by DNA/(DOGSHDO/DOPE). At the ratio of 1:8, cellular associated DNA of DOGSDSO/DOPE was slightly greater than that of DOGSHDO/DOPE treatment.While there was some differences in cellular associated plasmid DNA between the lipids and the ratio of lipid to plasmid, there was no correlation of the DNA to transgene expression (Figures 2-5 and 2-6).

Comparison of Gene Transfection Activity of DOGSDSO/DOPE with DOGSHDO/DOPE and DOTAP/DOPE

The ratio of cationic lipid to DNA is a crucial factor for effective gene transfer. Comparison experiments were done by using a fixed amount of DNA (3 μg/well). Transfection activity of six ratios of cationic lipid to DNA (1:1, 2:1, 3:1, 4:1, 8:1, and 10:1 (w/w)) were tested in COS 1 cells and CHO cells. Since the molecular weights of DOGSDSO and DOGSHDO are 971.64 D and 935.57 D respectively, the molar ratio or charge ratio of cationic lipid to DNA of DOGSDSO and DOGSHDO was similar. Transfection was studied both in serum free media (Figure 2-6) and growth media containing 10% of FBS (Figure 2-7).

As shown in Figure 2-6, with transfection in serum free media, both DOGSDSO and DOGSHDO demonstrated the highest transfection at the weight ratio of 10:1 or 8:1 of cationic lipid to DNA (charge ratio, 6/1(+/-) assuming the two amino groups of ornithine are ionized. Further increases in the amount of liposome caused a decrease in transfection (data not shown). DOTAP reached optimal transfection activity at the weight ratio of 1:1 or 2:1(charge ratio, 1/2, 1/1 (+/-)). At the optimal ratio of each liposome, the transfection activity of DOGSDSO was 11 times higher than its analog

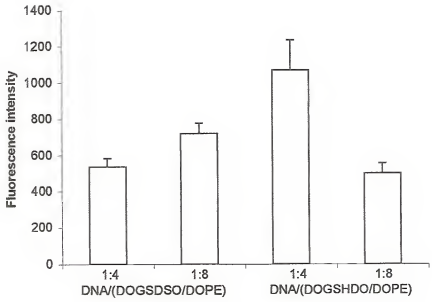


Figure 2-5: Cellular associated pDNA delivered by DOGSDSO/DOPE or DOGSHDO/DOPE. One μg/well of fluoresin-labled pDNA was complexes with DOGSDSO/DOPE or DOGSHDO/DOPE at the ratio of 1:4 or 1:8 and was transfected to SKnSH cells as in Methods. The cellular associated DNA was analyzed by using flow cytometry as stated in Methods.

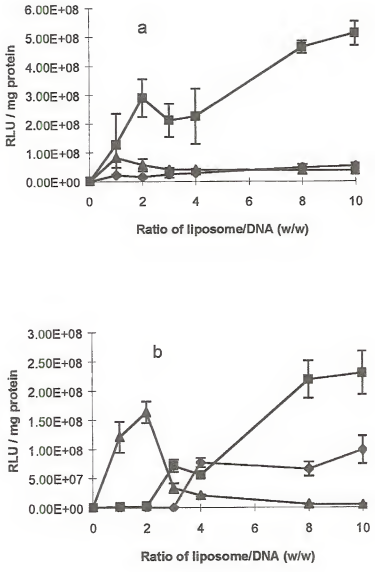


Figure 2-6: Comparison of the transfection of DOGSDSO/DOPE, DOGSHDO/DOPE, DOTAP/DOPE in serum free media. pGL3 DNA (3μg/well) was mixed with increasing amount of cationic liposomes (calculation based on cationic lipid) and used for transfection. a: COS 1 cells. b: CHO cells. ■: DOGSDSO/DOPE; ◆: DOGSHDO/DOPE, ▲: DOTAP/DOPE. Data is shown as mean ±S. D. (n=3).

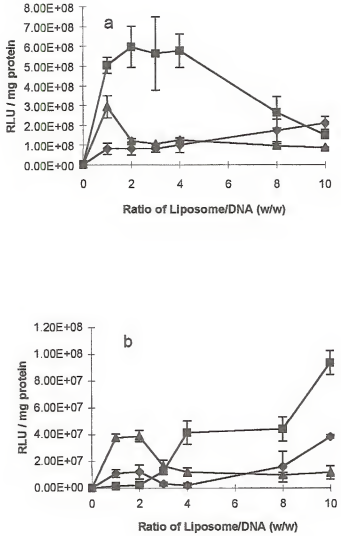


Figure 2-7: Comparison of the transfection activity of DOGSDSO/DOPE, DOGSHDO/DOPE, and DOTAP/DOPE in growth media containing 10% FBS. 3μg/well of pGL3 DNA were mixed with increasing amount of cationic liposomes and used for transfection. a: COS 1 cells. b: CHO cells. ■: DOGSDSO/DOPE; ◆: DOGSHDO/DOPE; ▲: DOTAP/DOPE. Data is shown as mean ± S. D. (n=3).

DOGSHDO and 6 times greater than the highest transfection activity of DOTAP (at the weight ratio 1:1 in COS11 cells (Figure 2-6a). In CHO cells, at the optimal ratio of each liposome, DOGSDSO showed 2.5 times higher transfection activity than DOGSHDO and 1.5 times higher than DOTAP (Figure 2-6b).

When pDNA was transferred in growth media containing serum (Figure 2-7), the optimal time used for incubation of cells with transfection complexes was 12 hours, which was optimized by another experiment (Figure 2-8). DOGSDSO demonstrated higher transfection activity in COS 1 (5.9×10^8 RLU/mg protein) (Figure 2-7a) and was comparable to the transfection activity in serum free media (Figure 2-6a). When the weight ratios of DOGSDSO to DNA were 1:1, 2:1, 3:1, and 4:1(w/w), the expression of luciferase protein was not statistically different ($p>0.05$). The highest transfection activity of DOGSDSO was 3 times higher than that of DOGSHDO and 2 times higher than that found in DOTAP in COS 1 cells (Figure 2-7a). In CHO cells, transfection was inhibited by serum, as the optimal transfection activity of all three liposomes in serum was three times lower than that of in serum free media. However, when compared with each other, the optimal transfection activity of DOGSDSO was still more than two times higher than the optimal transfection activity of DOTAP or DOGSHDO (Figure2-7b).

Transfection Activity of Liposomes as a Function of Time

When DNA transfection was carried out in serum free media, the DNA/liposome complexes were usually incubated with cells for 4-5 hours (Felgner et al. 1994, Gao and Huang 1991, Liu et al. 1996). This period of exposure was selected based on two observations: 1) it was reported that most of the complexes were internalized by 6 hours (Zabner et al. 1995), and 2) further increase of incubation time without serum leads to a

lack of growth nutrition, and thus cells are more vulnerable to the toxicity of complexes (Farhood et al. 1992). For gene transfection in growth media containing 10% FBS, the toxicity of complexes may be alleviated. On the other hand, the transfection may also be inhibited by enzymes or slowed down by negatively charged macromolecules. As shown in Figure 2-8, both the transfections of DOGSDSO and DOTAP reached the highest activity at 8-12 hour transfection time in CHO cells. Further increases in incubation time resulted in the decrease of transfection. The lower transfection may be caused by the cytotoxicity of the DNA/liposome complexes. However, the transfection activity of DOGSHDO was not different from 2 to 48 hour transfection periods. According to our observations, with DOTAP/DNA at the weight ratio of 4:1, transfection complexes formed large aggregates. The large aggregates of complexes may detrimentally affect further gene transfection (Liu et al. 1996).

GFP Gene Expression

The transgene expression of green fluorescence protein of DOGSDSO/DOPE, DOGSHDO/DOPE and DOTAP/DOPE were studied by using the optimal transfection ratio of each liposome in CHO cells (Figure 2-9). The efficiency of DOTAP, DOGSDSO, and DOGSHDO was 5±1%, 22±2%, and 6±1%, respectively. When the efficiencies of the liposomes were compared to each other, the results were consistent with those of luciferase gene expression experiments (Figures 2-6 and Figure 2-7). This result indicates that not only more transgene is expressed in total cells per well but it also occurs in a greater number of cells.

Transgene Expression as a Function of the Amount of DNA/liposomes

Transfection activity of different formulations of cationic liposomes are usually studied by fixing either the amount of DNA or the amount of cationic liposomes and changing the ratios of liposome to DNA (Felgner et al. 1994, Gao and Huang 1991, Liu et al. 1996). In these experiments, to test the gene delivery efficiency of liposomes, we fixed the weight ratio of DOGSDSO/DNA and DOGSHDO/DNA at their optimal ratio of 10:1 (Figure 2-6), and treated 2×10^5 COS 1 cells and CHO cells with increasing amount of DNA/liposome complexes. As shown in Figure 2-10a, the system reached saturation at 2 μg of DNA. Further increases of DNA from 2 μg to 7 μg, resulted in no significant increases in transgene expression. Contrary to the transfection with DOGSDSO, the transgene expression mediated by DOGSHDO increased with greater amount of complexes.

For the transfection in CHO cells, DOGSDSO also demonstrated better transfection efficiency than DOGSHDO (Figure 2-10b). The system reached saturation with 4 or 5 μg of DNA. There was no significant difference of gene expression between the treatments of 5 μg of DNA and 6 μg of DNA ($p>0.05$). A further increase of DNA to 7 μg resulted in a decrease of gene expression ($p<0.05$). Furthermore, gene expression continued to increase with the higher the amounts of DNA/DOGSHDO liposome. This trend was similar to that observed in COS 1 cells.

Discussion

In this study, a new strategy to synthesize disulfide bond-containing cationic lipids was reported. Compounds containing a disulfide bond are able to participate in

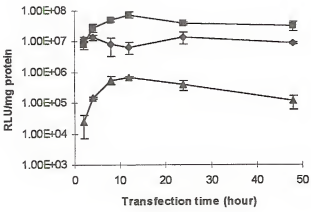
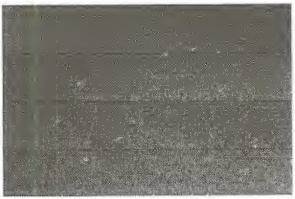
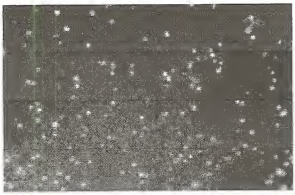


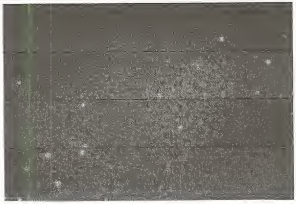
Figure 2-8: The effect of incubation time on the transfection activity. pGL3 DNA (3 μg/well) was mixed with 12 μg of DOGSDSO/DOPE, DOGSHDO/DOPE, and DOTAP/DOPE respectively and used for transfection in CHO cells. Transfection was done in growth media, and transfection mixtures were changed to fresh growth media after a set of incubation periods, respectively. Post-transfection incubation time was 24 hours. ■: DOGSDSO/DOPE; ◆: DOGSHDO/DOPE;▲: DOTAP/DOPE. Data is shown as mean ±S. D. (n=3).



(a)



(b)



(c)

Figure 2-9: Comparison of transfection of GFP gene by DOTAP/DOPE, DOGSDSO/DOPE, and DOGSHDO/DOPE in CHO cells. The optimal weight ratio for transfection of each liposome was used. Three μg per well of pUF2 DNA was mixed with 30 μg of DOGSDSO/DOPE, 30 μg of DOGSHDO/DOPE, and 6 μg of DOTAP/DOPE. Transfection efficiency was studied as indicated in Methods. Bright cells indicate cells that expressed green fluorescence protein (GFP). a: Transfection with DOGSHDO/DOPE, b: Transfection with DOGSDSO/DOPE; c: Transfection with DOTAP/DOPE.

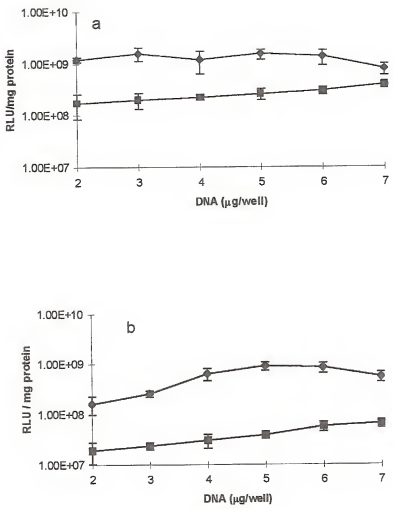


Figure 2-10: Effect of the amount DNA on transgene expression. Increasing amount of pGL3 DNA was mixed with DOGSDSO/DOPE or DOGSHDO/DOPE at the optimal transfection weight ratio of 10/1 and used for transfection as indicated in Methods. a: COS 1 cells; b: CHO cells. ◆ DOGSDSO/DOPE; ■: DOGSHDO/DOPE. Data is shown as mean ±S. D. (n=3).

disulfide exchange reactions over a broad range of conditions-from acidic to basic pH- and in a wide variety of buffer constitutes and physiological conditions (Hermanson 1996). Because of their special chemical properties, disulfide conjugate techniques have widely been used in drug delivery to achieve high delivery efficiency (Boutorine and Kostina 1993, Legendre et al. 1997, Trail et al. 1997, Trail et al. 1993). The normal method used in bioconjugation is involved in crossing-linking or modification reactions using disulfide exchange processes to form disulfide linkage with sulfhydryl-containing molecules. This method is not suitable for the syntheses of most cationic lipids for their specific chemical structures. We synthesized DOGSDSO by using a simple and convenient method without using cross-linking strategy. This method may be used to synthesize kinds of new cationic lipids which contain a disulfide linker. In this study, we demonstrated that DTT and cell lysate induced greater plasmid DNA release from disulfide cationic liposomes than non-disulfide containing liposomes. In addition, the results of cellular associated pDNA were not proportional to the transgene expression. Therefore, the enhancement of transfection by disulfide cationic liposomes was not resulting from additional DNA delivered by disulfide liposomes. This observation also indicates that there might be subcellular compartments (e.g., membrane surface, or within the cell endosomes, lysosomes) which are not in contact with the cellular machinery responsible for DNA processing. We systematically studied gene delivery of a lipid containing a disulfide bond (i.e., DOGSDSO) and compared it to its non-disulfide analog DOGSHDO and commonly used lipid, DOTAP. DOGSDSO demonstrated greater gene transfection in both COS 1 and CHO cells. In addition to its higher transfection activity

in the absence of serum (Figure 2-6), DOGSDSO was found equally active in the presence of 10% FBS in COS 1 cells (Figure 2-7a).

The barriers involved in gene delivery (Zabner et al. 1995) such as the formation of liposome/DNA complexes, entry of complexes into cells and escape of DNA from endosomes may be similar for DOGSDSO and DOGSHDO based on their similar physical properties. Another critical barrier in gene delivery is DNA's dissociation from the cationic lipid (Xu and Szoka 1996, Zabner et al. 1995). DNA must dissociate from liposomes before transcription can occur. Behr et al. demonstrated that no transgene expression was detected by direct intranuclear or intracytoplasmic injection of complexes of cationic liposomes (Pollard et al. 1998). Several groups have indicated that less than 15% of the cell-associated DNA is released into the cells (Felgner et al. 1987, Legendre and Szoka 1992). Szoka and colleagues proposed the release of DNA from the complexes by anionic lipids, and this four-step mechanism is postulated to be relatively slow (Xu and Szoka 1996). In the disulfide liposome/DNA complexes, the disulfide bond is unstable in the presence of high concentrations of intracellular reductive substances. The metabolism of the disulfide bond facilitates the dissociation of DNA as reported in the current studies. The release of DNA is one hypothesis explaining the differences in gene expression for the two lipids (i.e., DOGSDSO and DOGSHDO) although other answers may also coexist such as differences in cellular toxicity or disulfide activation of cellular machinery.

It is also interesting to note that the optimal ratio of DOTAP to DNA in transfection assays was 1:1 or 2:1 (w/w). Theoretically, at the weight ratios of 1:1 and 2:1, the charge ratio of the lipid-DNA complexes are about 1:2 and 1:1(+/-), respectively,

which means complexes at these weight ratios are negatively or neutral charged. These results seem to conflict with previous literature studies, which suggested that cationic lipid-mediated gene transfection is efficient when the lipid/DNA complexes have a net positive charge (Felgner et al. 1994, Gao and Huang 1991, Liu et al. 1996). However, when an agarose gel retardation was used to analyze the complexes of DOTAP/DNA at weight ratios of 1:1 and 2:1, it was found a relative large fraction of free pDNA (picture not shown) remained in the mixture. The zeta potential of DNA/DOTAP complexes at these two ratios was +0.07 mV and +0.78 mV respectively. These results suggest the charge of the complexes was different from that of theoretical calculations and the net charge of the complexes was still slightly positive. These findings support the conclusion that cationic-DNA lipid complexes are usually heterogeneous, and only a fraction of the complex may be effective in transgene delivery. It was also observed that at the weight ratios of DOTAP to DNA larger than 2:1, transfection complexes formed large aggregates during incubation. Although complexes were used for transfection within 5-10 minutes after DOTAP was mixed with DNA as previous study (Liu et al. 1996), complexes were still found to form large aggregates in half an hour during transfection. The lack of physical stability of liposome/DNA complexes affects the gene transfection activity (Caplen et al. 1995b). However, according to our observations, at the weight ratio of 10:1 (charge ratio 6/1(+/)), DOGSDSO and DOGSHDO did not aggregate during the transfection period. These findings may be due to the different lipid headgroups. DOTAP has a quaternary amino head group, while DOGSDSO and DOGSHDO have an ornithine head group which bears two primary amines. The interaction force between primary amino groups and DNA is not as strong as the interaction force of quaternary

amine with DNA. These physical binding characteristics of DOGSDSO and DOGSHDO are similar to that of 3-β-[N-(N', N'-dimethylaminoethanol) carbarmoyl] cholesterol (DC-Chol). As rationalized by Huang and colleagues, a weaker interaction between DNA and DC-Chol/DOPE may reduce the tendency for aggregation of DNA-liposome complexes, thus maximizing the production of complexes that are taken up efficiently by cells (Caplen et al. 1995b).

It is easily seen from Figures 2-6 and 2-7 that there are differences in the gene transfection profiles for the various liposomes between the two cell lines. DOGSDSO liposomes do not reach maximum activity until the highest tested weight ratio (i.e., 10:1 liposome/DNA). The difference in transfection activity was not due to a difference in cellular accumulation of DNA as indicted by flow cytometry experiment. When the studies are taken together they imply there is no correlation between cellular associated DNA and transgene expression. This finding could be explained by either a saturation of the cells machinery for handling plasmid DNA or a distinct subcellular compartment that contains the plasmid and, so that monitoring total cellular DNA amounts may not be adequate in developing new delivery strategies. Intriguing findings were observed in Figure 2-7 when the transfection was done in serum containing media. In COS cells the maximum activity of DOGSDSO was reached at a lower ratio of liposomes/DNA (i. e. 2-4) followed by a decline in activity at higher ratios although the total amount of transgene expression remained constant at the maximum levels. This finding may be explained by the deposition of serum proteins onto the complex leading to altered transport. This decreased expression was also observed with DOGSHDO liposomes. In CHO cells, similar shaped profiles were obtained for all liposomes tested regardless of the presence

of serum. The addition of serum resulted in significant decreases in transgene activity, which has been observed by other investigators. It is unclear why the two cell lines differ to such a large extent. This difference may be due to the transport properties of the particles formed or intrinsic differences in the cell lines (e.g., growth rate, transcriptional activity, and luciferase stability).

Figure 2-8 depicts the effect of incubation time on transgene expression in serum containing-media. From the graph it appears that lipids containing ornithine head groups obtain maximal transfection at 10 hours while DOTAP peaks earlier. For both types of headgroups, longer transfection time (i.e., exposure to the DNA/lipid complex) does not result in higher transgene activity. This finding suggests that the majority of transport of active transfection particles occurs early, and with continued time, the DNA is digested or the lipid/DNA particles are altered in a manner which does not favor endocytosis.

Transfection activity of different formulations of cationic liposomes is usually studied either by fixing the amount of DNA or fixing the amount of cationic liposomes and changing the ratios of liposome to DNA(Felgner et al. 1994, Gao and Huang 1991, Liu et al. 1996). The transgene expression results are roughly bell shaped curves. It is generally agreed that the decreased activity of low lipid/DNA ratios is due to reduced cell membrane binding or greater metabolism, and the decline in transfection activity at high ratios reflects toxic effects of cationic lipid (Vigneron et al. 1996). The correlation of amount of DNA/liposome complexes with transgene expression is also an important aspect in reflecting of their transfection efficiency. As shown in Figure 2-9, at the optimal ratio of DNA/liposome, 2 μg of DNA transferred by DOGSDSO reached the saturation of the system in COS 1 cells. Further increases in dose exhibited no significant

increase in luciferase activity. However, 7 μg of DNA transferred by DOGSHDO did not reach saturation, and the transgene expression was only one third that of 2 μg of DNA transferred by DOGSDSO liposomes. This finding infers that different amounts of free DNA may be reaching the cytoplasm. This result suggests it is important to use a concentration of DNA that will not saturate the intracellular enzymatic processing of DNA when making comparisons in delivery vectors. These characteristics of DOGSDSO will be useful in its future application in animal studies or in clinical trials. The toxicity of cationic liposomes is one of the drawbacks of cationic liposome-mediated gene transfer, and highly efficient vectors with relatively small dose are needed in future clinic applications.

In summary, liposomes made of DOGSDSO/DOPE could be degraded by cell lysates in a manner that is postulated to enhance the release of plasmid DNA to produce free DNA for transcription. DOGSDSO/DOPE had a higher transfection activity and transgene expression efficiency than its analog DOGSHDO/DOPE or DOTAP/DOPE liposomes. Disulfide bond-containing cationic liposomes may release DNA from complexes more easily than normal lipids, and thus increase gene expression. Our results also indicate that when comparing various delivery systems or plasmid constructs, it is important to characterize the cell line being studied to ensure that you are in the linear portion of the dose response curve.

CHAPTER 3
USE OF DITHIODIGLYCOLIC ACID AS A TETHER FOR CATIONIC LIPIDS DECREASES THE CYTOTOXICITY AND INCREASES TRANSGENE EXPRESSION OF PLASMID DNA *IN VITRO*

Introduction

Cationic lipids are popular non-viral gene transfer vectors (Felgner et al. 1995, Gao and Huang 1995, Rolland 1998). However, two major barriers for cationic lipid-mediated gene transfer limit the application of cationic lipids. Firstly, the low transfection efficiency compared to the virus system, and secondly, the toxicity. Since Felgner et al firstly reported application of N-[1-(2,3-dioleyloxy)propyl]-N, N, N-trimethylammonium chloride (DOTMA) in transfection of plasmid DNA in 1987 (Felgner et al. 1987), many cationic lipids have been synthesized and used in plasmid DNA delivery (Gao and Huang 1995, Lee and Huang 1997, Rolland 1998). Most of these efforts have been focusing on improving the transfection activity and decreasing the cytotoxicity. The uses of ester, amide, and carbamate linkages to tether polar and hydrophobic domains are common strategies to lower the toxicity. However, no intracellular degradation studies have been conducted for any cationic transfection lipid (Aberle et al. 1998). 3-β-[N-(N', N'-dimethyl amino ethane) carbamoyl] cholesterol (DC-Chol) was the first lipid used in clinical trials because of its combined properties of transfection efficiency, stability and low toxicity (Gao and Huang 1995). Recently, Nantz et al reported a novel tetraester construct that reduced the cationic lipid-associated cytotoxicity compared to DC-Chol (Aberle et al. 1998). However, the introduction of the

ester bond may also decrease the stability of liposome in systemic circulation when the liposomes are used in clinical trials. A strategy is proposed to take advantage of the high intracellular reductive environment to make a disulfide linker-containing cationic lipid, which is stable outside cells whereas it can be reduced in cells by intracellular reductive substance (Tang and Hughes 1998). The reduction of the disulfide bond will enhance the release of DNA from DNA/liposomes complexes. Dissociation of DNA from DNA/liposomes complexes is one of the major barriers for cationic liposome-mediated gene transfection (Escriou et al. 1998a, Rolland 1998, Zabner et al. 1995). Previously, we reported that a disulfide lipid - 1', 2'-dioleoyl-sn-glycero-3'-succinyl-2-hydroxyetheyl disulfide ornithine conjugate (DOGSDSO) could increase the plasmid gene transfection compared to its non-disulfide cationic lipid. However, the toxicity of the lipid was not significantly altered by the addition of disulfide bond (Tang and Hughes 1998). We demonstrated that dithiothreitol (DTT) could reduce the part of disulfide cationic lipid and help to release part of DNA from DNA/liposomes complexes. However, DTT is not a cellular substance. When we tried to use a cellular reductive substance (e.g., glutathione) in the same experiment, no DNA was released. DTT is a stronger reductive reagent than glutathione since it forms a stable six member-ring product (Hermanson 1996). We hypothesized that the disulfide linker in DOGSDSO might be too strong so that it could not be degraded fast enough to significantly decrease the cytotoxicity. Since the disulfide bond of dithiodiglycolic acid is weakened by the strong electric withdrawing effects of two carboxyl groups which are symmetrically connected to the two α-carbons of disulfide bond (Figure 3-1), it was hypothesized that introduction of dithiodiglycolic acid to tether the polar group and lipophilic domains will increase the

transfection activity and decrease the toxicity of cationic lipid. Also the favorable results obtained with DC-Chol *in vivo* demonstrated that cholesterol is a suitable backbone as a starting reagent for cationic lipids. We synthesized cholesteryl hemidithiodiglycolyl tris(aminoethyl)amine (CHDTAEA) with a dithiodiglycolyl linker, its nondisulfide anonlog cholesteryl hemisuccinyl tris(aminoethyl)amine (CHSTAEA). We compared their transfection activities and cytotoxicity of CHDTAEA, CHSTAEA and DC-Chol in mammalian cells. The results demonstrated dithiodiglycolic acid as a linker in cationic lipid can greatly increase the transfection activity and decrease the toxicity of cationic lipids.

Materials and methods

Syntheses of CHDTAEA and CHSTAEA

CHDTAEA was synthesized as outlined in Figure 3-2. 2.0 gram (6 mmol) of cholesterol and 1.9 gram of dithiodiglycolic acid (12 mmol) were dissolved in 30 ml of ethyl acetate under nitrogen. 12 mmol of 1,3-Dicyclohexylcarbodiimide (DCC), 0.19 gram of 4-pyrrolidinopridine (1.2 mmol), and 1.43 ml of triethylamine (12 mmol) were added at 0 ^{0}C. The mixture was warmed to room temperature naturally and stirred under nitrogen at room temperature overnight. The reaction mixture was filtered and washed by 5% citric acid followed by brine three times. The organic layer was separated and dried over anhydrous sodium sulfate. The organic solvent was evaporated. The product cholesteryl hemidithiodiglycolate was purified on silica gel with a developer of hexane:ethyl acetate:acetic acid (30:10:1) (R_f=0.3, 2.4 gram, yield=80%). N, N-BOC_2-tris (2-aminoethyl) amine was prepared by treating tris(2-aminoethyl)amine with two

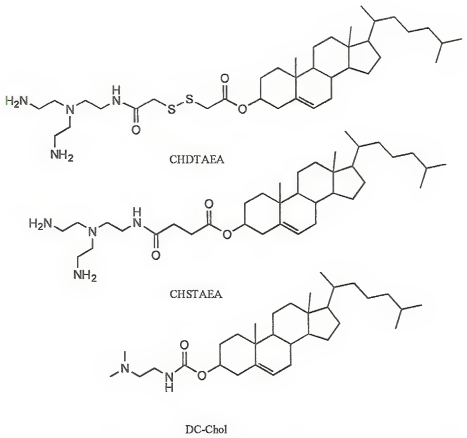


Figure 3-1: Structures of cholesteryl N-(dimethylaminoethyl)carbamate (DC-Chol), cholesteryl hemidithiodiglycolyl tris(aminoethyl)amine (CHDTAEA), and cholesteryl hemisuccinyl tris(aminoethyl)amine (CHSTAEA).

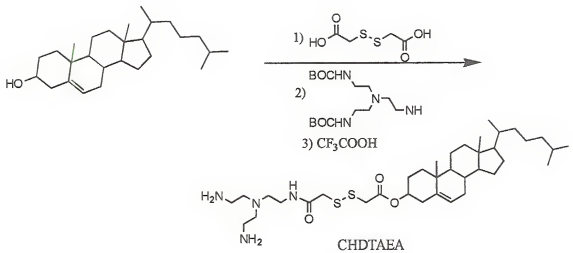


Figure 3-2: Scheme of synthesis of Cholesteryl hemidithiodiglycolyl tris(aminoethyl)amine (CHDTAEA).

equivalent of 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON) in wet THF. 0.30 gram (0.5 mmol) of cholesteryl hemidithiodiglycolate, 0.24 gram of N, N-BOC_2-tris(2-aminoethyl)amine (0.65 mmol), and 0.007 gram of 4-pyrrolidinopridine (0.005 mmol) were dissolved in 20 ml of dichloromethane. 0.6 mmol of DCC in dicholormethane and 0.06 ml of triethylamine were added dropwise at 0 ^{0}C. The mixture was stirred under nitrogen at room temperature overnight. The product N, N-BOC_2-(cholesteryl hemidithiodiglycolyl tris(aminoethyl)amine) was separated and purified on silica with a developer of dichlorometnae:methanol (10:1) (Rf=0.35, 0.37 gram, yield =85%). The molecular weight was determined by a Micromass Quattro-LC-Z triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) ion source (Beverly, MA). $M+H^+$= 881.5 (calculated 881.3 for $C_{47}H_{82}N_4O_7S_2+H^+$). ^{1}H NMR ($CDCl_3$, 300 MHZ): 0.69 [s, CH_3], 0.830-2.04 [m, 3×(CH_3), 10×(CH_2), and 6×(CH)], 1.02 [s, CH_3], 1.42 [s, 2×$(CH_3)_3CO$], 2.38 [m, CH_2C=C], 2.52-2.68 [m,$(CH_2)_3N$], 3.28 [m, 2×(CH_2NCO)], 3.35 [m, CH_2NCO], 3.50 [s, CH_2SS], 3.60 [s, CH_2SS], 4.68 [m, CHO], 4.99-5.32 [m, 3×NHCO], 5.38 [m, C=CH]. BOC groups were detached (CF_3COOH, 0 ^{0}C, 15 min), and excessive trifluoroacetic acid was dried under high vacuum to produce CHDTAEA. This step is quantitative as reported by Behr (Behr et al. 1989). After the reaction, the purity was checked by TLC, CHATAEA were used to prepare liposomes without further purification. Similar synthesis strategy was used to synthesize the non-disulfide analog CHSTAEA (Fig.1) with slight modifications. Commercial available cholesteryl hemisuccinate was directly used to conjugate with N, N-BOC_2-tris (2-aminoethyl)amine to obtained the BOC protected CHSTAEA. $M+H^+$= 817.5 (calculated 817.2 for $C_{47}H_{82}N_4O_7+H^+$). ^{1}H NMR

($CDCl_3$, 300 MHZ): 0.69 [s, CH_3], 0.830-2.04 [m, 3×(CH_3), 10×(CH_2), and 6×(CH)], 1.02 [s, CH_3], 1.44 [s, 2×$(CH_3)_3CO$], 2.30 [m, $CH_2C{=}C$], 2.48-2.70 [m,$(CH_2)_3N$], 3.15 [m, 2×(CH_2NCO)], 3.29 [m, CH_2NCO], 4.61 [m, CHO], 4.99-5.32 [m, 3×NHCO], 5.35 [m, C=CH]. The same method was applied to detach the BOC group as mentioned above.

Liposome Preparation

CHDTAEA or CHSTAEA was dissolved in chloroform and mixed with a neutral helper lipid DOPE [1:1 molar ratio, which was optimized for transfection (data not shown)]. DC-Chol was dissolved in chloroform and mixed with DOPE at the optimal molar ratio of 6:4 according to Gao and Huang (Gao and Huang 1991). The mixtures were evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The lipid film was dried by nitrogen for a further ten minutes to evaporate residual chloroform and the lipid film was resuspended in sterile water to a concentration of 1 mg/ml based on the weight of cationic lipids. The resultant mixtures were shaken in a water bath at 30 ^{0}C for 30 minutes. The liposomes were hydrated at 4 ^{0}C overnight. The suspensions were sonicated using a Sonic Dismembrator (Fisher Scientific) for 2 minutes at 4 ^{0}C to form homogenized liposomes. The particle size distribution and zeta potential of liposomes were measured using a NICOMP 380 ZLS instrument (Santa Barbara, CA). The diameters were expressed by the volume-weight distribution parameter. The average diameters of CHDTAEA/DOPE, CHSTAEA/DOPE and DC-Chol/DOPE were 121.7 ± 14.2 nm, 154.7± 19.8 nm, and 165.8± 19.9 nm, respectively. The zeta potentials were + 8.04 mV, +7.68 mV, and +6.98 mV, respectively. Liposomes were stored at 4 ^{0}C and used in 3 months.

Gel Electrophoretic Analysis of Release of DNA from DNA/liposome Complexes by Glutathione

One μg of plasmid DNA was dissolved in 10 μl of phosphate-buffer saline (PBS). Cationic liposomes CHATAEA/DOPE or CHSTAEA/DOPE were added to pDNA solution at the increasing weight ratios of 2/1, respectively. All calculations of the weight of liposomes in this paper were based on the weight of cationic lipids only. After the complexes were incubated for 30 minutes at room temperature, 10 μl of 20 mM of glutathione in PBS (pH=7.3) was added to the mixture to reach a final concentration of glutathione of 10 mM. The mixtures were incubated at 37 ^{0}C for 20 hours. Released DNA was visualized by 1 % agarose gel electrophoresis.

Cell Culture and Luciferase Gene Transfection

Chinese hamster ovary (CHO) and SKnSH cells were obtained from American Tissue Type Collection (Rockville, MD). The cells were maintained in RPMI 1640 or α-MEM media respectively. All cells were kept in 5 % CO_2 and at 37 ^{0}C in humidified air. Cells of same passage from same flask were cultured and seeded in three 24 well plates (2×10^5 cells/well) and grown to 60-80 % confluence in 1 ml of serum-containing media. pGL3 luciferase DNA/liposome complexes were made and incubated at room temperature in serum free media for 30 minutes and used for transfection. The plasmid was isolated as previously described (Tang and Hughes 1998). Before transfection, growth media containing serum was changed to 400 μl serum free media. DNA/liposome complexes in 100 μl of serum free media were added to each well and incubated for 4.5 hours. After the 4.5-hour transfection time, the transfection media were changed to growth media and cells were incubated for another 24 hours. Luciferase activity was measured using a standard luciferase assay (Thierry et al. 1997). Luciferase

activity in transfected cells was expressed as RLU per well. All conditions for transfection were strictly kept consistent for each different kind of liposomes. Experiments were repeated at least twice, and experiments were done in triplicate within a given series.

Toxicity Assay

The toxicity of CHDTAEA/DOPE, CHSTAEA/DOPE and DC-Chol/DOPE in the CHO and SKnSH cell line was determined using a CARE-LASS (a calcein release assay) (Lichtenfels et al. 1994). Before the assay, cells were plated in a 96 well plate (1×10^5cells/well) and grown to 60-80 % confluence overnight. To test the toxicity of the DNA/liposome complexes, a fixed amount of DNA (1 μg/well) was used to complex with increasing amount of liposomes. The ratios of DNA to liposome of different treatments were the same as the ratios used in transfection studies. The cytotoxicity of three liposomes were measured and expressed according to our previous procedure (Tang and Hughes 1998). Each experiment was performed in triplicate and repeated at least twice.

Flow Cytometry Analysis of the Delivery of Fluorescein Labeled pDNA into CHO Cells

The nucleic acid fluorescein labeling kit was purchased from PanVera Corporation (Madison, WI), and pDNA was labeled according to the product directions. One μg/well of fluorescein labeled pDNA was complexed with CHDTAEA/DOPE, CHSTAEA/DOPE, or DC-Chol/DOPE with the weight ratio of cationic lipid to DNA of 2/1, respectively. The complexes were used for transfection as stated in preceding methods in CHO cells. After 4.5-hour transfection period, the cells were quickly washed with PBS three times. One hundred μl of trypsin-EDTA was added to each well. The

cells were trypsinized for 2-3 minutes and the reaction was stopped by 200 μl of 1% of bovine serum albumin (BSA) in PBS. The cells were collected and centrifuged at 250 g for 10 minutes. The supernatant was decanted and the cell pellet was resuspended in 500μl of 1% BSA and centrifuged for another 10 minutes to further wash phenol red and possible residue pDNA. After the BSA solution was discharged, the cell pellet was resuspended in 500 μl of PBS followed by 500 μl of 2% of paraformaldehyde PBS solution. The cells were fixed at 0 ^{0}C for 15 minutes then stored at 4 ^{0}C overnight. Cell fluorescence was measured using a Becton Dickinson FACSort flow cytometer (San Jose, CA). 10,000 cells were counted for each treatment. Cells, which were treated with non-labeled DNA/liposome complexes were used in compensation experiments. Cells were gated with their morphological properties. The mean fluorescence intensity of the cells was measured in terms of arbitrary units using a LYSYS II software program (Becton Dickinson, San Jose, CA). Each experiment was performed in triplicate and repeated twice.

Analysis of the Fate of Complexes in Transfection Using Confocal Microscopy

DNA/liposome complexes were doubled labeled. pDNA was labeled as stated previously. Liposomes were labeled by incorporating 1 molar percent of Rh-PE in liposomes. CHO cells were seeded on coverslips in 6-well plates at a density of 1×10^{5} cells/well and incubated overnight before transfection. Complexes of DNA and liposomes were administered to CHO cells as stated in transfection procedures. At the appropriate time points, serum free media (for 1-hour and 3-hour time points) or growth media (for 10-hour time period) were aspirated, and cells were washed with PBS twice. The cells were incubated with 1 ml of CellSrub^{TM} (washing) buffer (Gene Therapy

System, San Diego, CA) for 10 minutes, to remove the complexes attached on the cell surface and then washed by PBS. The cells were fixed in 2 percent paraformaldehyde at 4 ^{0}C and stored at 4 ^{0}C overnight before observation. A Bio-Rad MRC 1000 confocal microscope was used to observe the complexes in cells. An argon gas laser with excitation lines at 488, 568, and 647 nm was used to induce fluorescence. Excitation of the green fluorophores was achieved by using the 488 nm excitation lines, with the resulting fluorescent wavelengths observed by using a 515-540 nm band pass filter. Red fluorescence was induced by the 568 nm excitation line and detected as 575-640 nm wavelengths. The cells ranged in thickness from 2-8 μm, and confocal sections were typically taken every 0.2 μm.

Statistical Analysis

Statistical analyses were were performed using ANOVA-Fisher's PLSD Post hoc tests (Stat View 4.53, Abacus Concepts, Inc., Berkeley, CA). One-way ANOVA was applied to compare transgene expression or toxicity between different liposomes/DNA ratios of the same liposome. Two-way ANOVA was utilized among treatments of liposome gorups and liposomes/DNA ratios. A probability of less than 0.05 was considered significantly different.

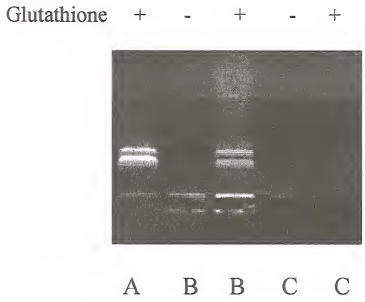


Figure 3-3: Gel electrophoretic analysis of the release of pDNA from cationic liposomes/DNA complexes in 10 mM glutathione in phosphate buffered saline. A: DNA, B: CHDTAEA/DOPE+DNA (2/1,w/w), C:CHSTAEA/DOPE+DNA(2/1,w/w).

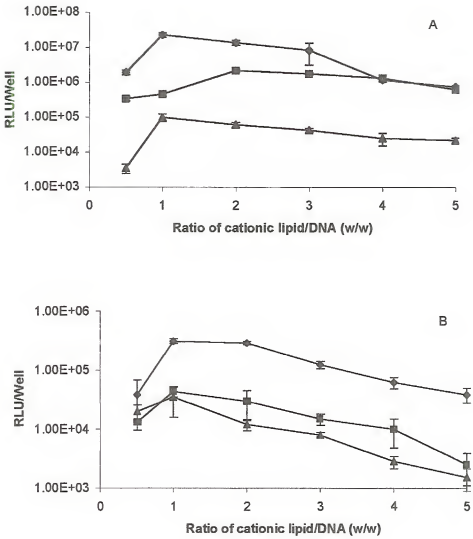


Figure 3-4: Comparison of the transfection of pGL3-luciferase plasmid by CHDTAEA/DOPE, CHSTAEA/DOPE, and DC-Chol in CHO and SKnSH cells. A fixed dose 2 μg/well of pGL3 DNA was mixed with increasing weight ratios of cationic liposomes (calculation based on cationic lipid) and used for transfection. A: Transfection in CHO cells, B: Transfection in SKnSH cells. ◆: CHDTAEA/DOPE, ■: CHSTAEA/DOPE; ▲: DC-Chol/DOPE. Data is shown as mean ±S. D. (n=3).

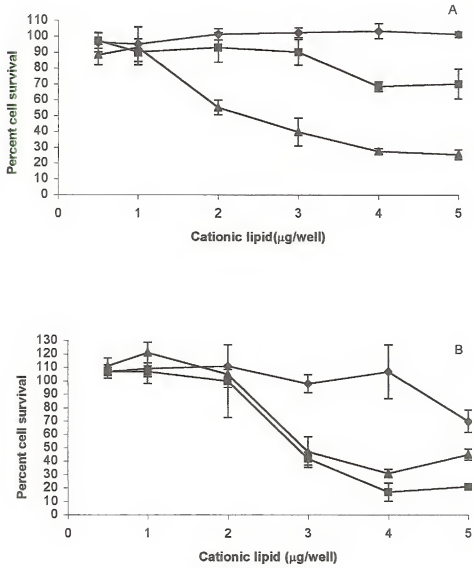


Figure 3-5: Toxicity assays. Cytotoxicity of the liposome/DNA complexes was studied in CHO and SKnSH cells. A fixed dose of 1 μg/well of pDNA was mixed with increasing amount of cationic liposomes and used in the toxicity assay. Cell viability was calculated as percentage of survival cells as stated in Methods. A: Toxicity in CHO cells. B: Toxicity in SKnSH cells. ◆: CHDTAEA/DOPE, ■: CHSTAEA/DOPE; ▲: DC-Chol/DOPE. Data is shown as mean ± S. D. (n=3).

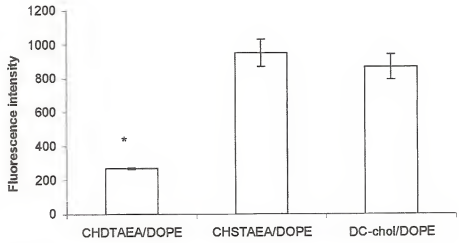
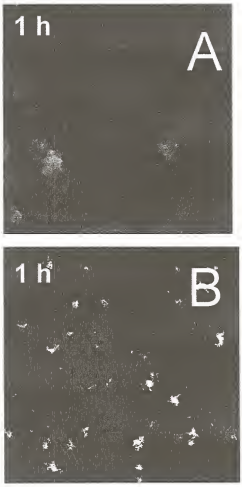
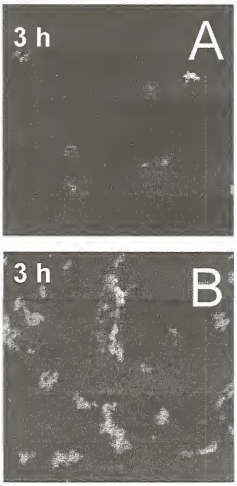


Figure 3-6: Comparison of the delivery of pDNA by CHDTAEA/DOPE, CHSTAEA/DOPE, and DC-Chol/DOPE in CHO cells. One μg/well of fluorescein labeled pDNA was complexed with CHDTAEA/DOPE, CHSTAEA/DOPE, or DC-Chol/DOPE with the weight ratio of cationic lipid to DNA of 2/1, respectively. The relative amount of pDNA, which was delivered into cells by different cationic liposomes, was measured as relative mean fluorescence intensity of cells as indicated in Methods. Data are shown as mean ±S. D. (n=3). *: Fluorescence intensity was different form other treatments ($p<0.05$, one-way ANOVA between treatments).



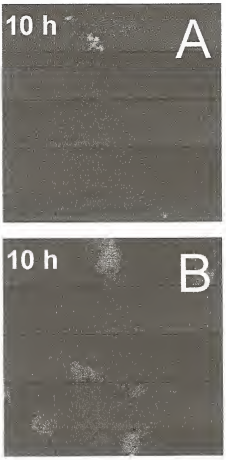
(a)

Figure 3-7: The comparison the fate of complexes of DNA/CHDTAEA/DOPE and DNA/CHSTAEA/DOPE in transfection of CHO cells. DNA and liposomes were labeled as stated in Methods and administered to cell cultures. The fate of complexes was observed using confocal microscope as in Methods. A: DNA/CHDTAEA/DOPE, B: DNA/CHSTAEA/DOPE. (a): one hours after complexes were administered in cells, (b): three hours, (c): ten hours.



(b)

Figure 3-7—continued.



(c)

Figure 3-7—continued.

Results

Release of DNA from Disulfide Cationic Liposomes/DNA Complexes by Glutathione

In this experiment, DNA was complexed with a disulfide cationic liposome CHDTAEA/DOPE or its non-disulfide cationic liposome analog CHSTAEA/DOPE at the cationic lipid to DNA ratio of 2/1. As indicated in Figure 3-3, DNA was tightly complexed by both cationic liposomes. In the absence of glutathione, disulfide liposomes/DNA complexes did not release DNA after 20-hour incubation in PBS at 37 ^{0}C. In the presence of 10 mM of glutathione, >50% of DNA was released from complexes after the incubation. In both reductive and non-reductive environments, non-disulfide liposome CHSTAEA/DNA complexes did not release any DNA. The results suggested cationic liposome/DNA complexes were fairly stable, and that cellular reductive substance (i.e. glutathione) can destabilize the complexes and help DNA to dissociate from liposomes. The complexes with the ratios of cationic liposome/DNA from 3/1 to 5/1 were also used to do the release assay with similar same results (data not shown).

Transfection of pGL3-luciferase Plasmid in Mammalian Cells

Transfection activities of liposomes of CHDTAEA/DOPE, CHSTAED/DOPE, and DC-Chol/DOPE were compared in CHO and SKnSH cells (Figure 3-4). The experiment conditions were strictly controlled. The same cell lines with the same passage number and from the same flask were plated in three plates. The cells were transfected and the transgene expression was analyzed at the same time. In CHO cells, the transfection activities of liposomes CHDTAEA/DOPE and DC-Chol/DOPE peaked at the weight ratio of cationic lipid to DNA of 1/1, and CHSTAEA/DOPE peaked at the

weight ratio of 2/1 (Figure 3-4A). At the optimal ratios of each liposome, CHDTAEA/DOPE had more than 100 times higher transfection activities than DC-Chol/DOPE, and 7 times higher transfection activities than its non-disulfide analog CHSTAEA/DOPE. In SKnSH cells, all three transfection agents peaked at weight ratio of 1/1. CHDTAEA/DOPE had 7 times greater transfection activities than both CHSTAEA/DOPE and DC-CHOL/DOPE (Figure 3-4B).

Cytotoxicity of Liposome/DNA Complexes

The cytotoxicity of DNA/liposome complexes was tested in both CHO and SKnSH cells. Cell viability was variable between CHO cells and SKnSH cells (Figure 3-5). As indicated in Figure 3-5A, CHDTAEA/DOPE was not toxic to CHO cells up to the dose of 5 μg/well ($p>0.05$) (Figure 3-5A). The non-disulfide analog CHSTAEA/DOPE had higher toxicity than CHDTAEA/DOPE at the doses of 4 μg/well and 5μg/well ($p<0.05$). DC-Chol/DOPE had only about 30 % cell survival at the dose of 5 μg/well. In SKnSH cells, CHDTAEA/DOPE also demonstrated lower toxicity, and it did not show significant toxicity until to the dose of 5 μg/well ($p>0.05$). At the dose of 5 μg/well, about 70 % of cells survived for the toxicity of CHDTAEA/DOPE. CHSTAEA/DOPE and DC-Chol/DOPE had much higher toxicity than CHDTAEA/DOPE at the doses of 3 μg/well, 4 μg/well, and 5 μg/well ($p<0.05$). In both cell lines, the data suggested that application of dithiodiglycolic acid as a linker could greatly decrease the toxicity of cationic liposomes.

Cellular Associated DNA by Different Liposomes

To investigate if the greater transgene expression by CHDTAEA/DOPE liposomes was resulted from greater amount of delivered pDNA by the liposomes, cell

associated fluorescein-pDNA after 4.5-hour transfection period was analyzed by using flow cytometric analysis. The relative amount of fluorescein-pDNA associated with cells after transfection period in CHO cells was measured by relative fluorescence intensity using flow cytometry. The weight ratio of cationic lipid to DNA of 2/1 was used for each liposome (i.e. CHDTAEA/DOPE, CHSTAEA/DOPE, and DC-Chol/DOPE) (Figure 3-6). It was suprising to find that CHDTAEA/DOPE liposomes delivered less DNA to cells than either the CHSTAEA/DOPE or DC-Chol/DOPE liposomes even though it had more that 100 times higher transgene expression than DC-Chol/DOPE and 7 times higher than CHSTAEA/DOPE at the weight ratio (i.e., 2/1) (Figure 3-4A). It is also worth noting that the cell associated pDNA of CHSTAEA/DOPE liposome and DC-Chol/DOPE liposome treatments were not significantly different whereas the transfection activity of CHSTAEA/DOPE was greater than that of DC-Chol/DOPE (Figure 3-4A).

The Fate of DNA/liposome Complexes in Transfection

As shown in Figure 3-7a, after1-hour transfection period, complexes were mainly attached to cell membrane and faint signal were seen in cells. The signal of CHSTAEA liposomes treated cells were much strong than that of CHDTAEA treated cells. Figure 3-7b shows that strong fluorescence signal were seen in both CHDTAEA and CHSTAEA liposomes treated cells. Similar to Figure 3-7a, CHSTAEA treated cells had more fluorescence intensity than that of CHDTAEA treated cells after 3-hour transfection period. After 10-hour period, the fluorescence signal in CHDTAEA liposomes treated cells faded away (Figure 3-7c). However, intensive fluorescence were still seen in CHSTAEA liposomes treated cells.

Discussion

The use of ester, amide, and carbamate linkages to tether polar and hydrophobic domains is a common strategy to lower the toxicity of cationic lipids. However, the introduction of an ester also sacrifices the stability of cationic lipids because of hydrolysis reaction in the aqueous extracellular environment. The application of disulfide as a linker in cationic lipid is an alternative strategy to decrease the toxicity and increase the transfection activity of cationic lipids. The disulfide is stable at non-reductive extracellular environments and is unstable in the presence of high concentration of reductive intracellular substance, e.g. the concentration of glutathione in human tissue such as liver and lens is up to 10 mM (Halliwell 1988). Disulfide conjugate techniques have widely been used in drug delivery to achieve high delivery efficiency (Boutorine and Kostina 1993, Legendre et al. 1997, Trail et al. 1997) in the development of treatments for cancer. The normal method used involves crossing-linking or modification reactions using disulfide exchange processes to form disulfide linkage with sulfhydryl-containing molecules (Boutorine and Kostina 1993, Legendre et al. 1997, Trail et al. 1997). This method will result in the more stable disulfide compound. A reductive substance such as dithiothreitol (DTT) is usually used to prove the hypothesis that the disulfide conjugate linker can be broken after delivery of conjugated drug *in vivo*. However, DTT is not a cellular substance. In this study, we used a cellular reductive substance (i.e. glutathione) to reduce the disulfide and release the complexed DNA. In CHDTAEA lipid, the disulfide bond is weakened by the strong electric withdrawing effects of two carboxyl groups, which is symmetrically connected to the two α-carbons of disulfide bond (Fig. 1). CHDTAEA liposomes released more than half of complexed

DNA in the presence of glutathione (Figure 3-3). As shown in Figure 3-3, CHDTAEA was as stable as its non-disulfide analog CHSTAEA in a non-reductive environment since DNA/CHDTAEA/DOPE liposomes did not release any pDNA after incubation. Therefore, the introduction of this weak disulfide bond did not sacrifice the stability of DNA /liposome complexes outside cells. Without a disulfide bond, its analog CHSTAEA did not release DNA at any conditions after incubation for 20 hours. The release of DNA from DNA/liposomes complexes is a major barrier for cationic lipid-mediated gene transfection (Escriou et al. 1998a, Rolland 1998, Zabner et al. 1995). An interesting finding was observed during this study, very little DNA was released at the early time points of incubation until about 20 hours (data not shown), after this time a large portion of the DNA was released. This result implies that a certain fraction of the liposome has to be degraded before the plasmid is released. More DNA released from the delivery system should result in greater transgene expression if the transcription machine has not been saturated.

The results of transgene expression demonstrated that CHDTAEA liposomes had greater transfection activity than its non-disulfide analog CHSTAEA in both CHO and SKnSH cells. A second major concern is the toxicity of the delivery system. Less toxic agent may have greater transfection. An optimal cationic lipid should be able to complex DNA and deliver DNA into cells, then release DNA from complexes for transcription (Pollard et al. 1998, Zabner et al. 1995). Finally, cationic liposome should be quickly degraded otherwise freed liposome will fuse with cell membrane and result in cytotoxicity. In a dithiodiglcolyic acid linker, the disulfide is relative weak and can be easily degraded by cellular substance such as glutathione. This facet of the lipid should

lower toxicity, increase the amount of free plasmid DNA within the cell and ultimately both of these event should increase transfection. As shown in Figure 3-5, in CHO cells, complexes of DNA/CHDTAEA/DOPE did not show cytotoxicity at all tested concentrations ($p>0.05$). In SKnSH cells, it did not show cytotoxicity until at the concentration of 5 μg/well. Without the dithiodiglycolic acid linker, CHSTAEA and DC-Chol had much greater cytotoxicity (Figure 3-5). To investigate whether the greater gene transfection of CHDTAEA/DOPE liposomes resulted from more DNA delivered into cell instead of released into the cytosol, we investigated the cell associated DNA after transfection period. Although CHDTAEA/DOPE had greater transfection activity, it had less cellular associated DNA than CHSTAEA/DOPE and DC-Chol ($p<0.05$). Confocal microscope data showed that fluorescence signal of complexes of DNA with disulfide liposomes in transfected cells faded away much faster than complexes of non-disulfide lipsomes. Two possible reasons might exist. First, the DNA/disulfide liposome complexes collapsed due to the decomposition of disulfide bond. The disintegration of complexes led to the dilution of fluorescence signal. Second, the fluorescence tag of DNA lost faster without the protection of liposomes after disulfide liposomes were degrade in cells. Whether the fast release of DNA from liposomes would result in short term transgene expression is an interesting question. To address this issue, we conducted 2 to 6-day transfection experiments. CHDTAEA liposomes transfected cells had greater transgene expression for up to 6 six days than that of CHSTAEA treated cells (data not shown) although fluorescence signal of complexes faded away in 10 hours (Figure 3-7c). The results supported the previous hypothesis that the association of DNA from DNA/cationic liposomes is a one of the major barrier for gene delivery.

In summary, the application of dithiodiglycolic acid as a linker to tether polar domain of lipophilic domain of cationic lipid can enhance the transfection activity and decrease the cytotoxicity of cationic lipids. Dithiodiglycolic acid may be used in synthesizing new cationic lipids and may also be used as a drug conjugation linker.

CHAPTER 4
SYNTHESIS OF A SINGLE-TAILED CATIONIC LIPID AND INVESTIGATION OF ITS TRANSFECTION

Introduction

Many cationic lipids have been synthesized and used for gene therapy in cell culture, animals, or clinical trials (Gao and Huang 1995, Lee and Huang 1997, Mahato et al. 1997, Rolland 1998), since a cationic lipid was reported to be used in plasmid delivery by Felgner's group in 1987 (Felgner et al. 1987). It is very hard to predict the gene transfection activities of synthetic cationic lipids due to the poor understanding of the mechanism of cationic lipid-mediated gene transfection. All cationic lipid molecules contain three functional domains: a positive charged head group, a hydrophobic region, and a linker that tethers the cationic group and hydrophobic groups. Two major types of hydrophobic moieties are used in cationic lipids: one is a pair of aliphatic chains, and the other is based on cholesterol. Huang et al. reported single-tailed cationic lipids, such as cetyl trimethylammonium bromide (CTAB), to be more toxic and less efficient than their double-tailed counterparts, such as N- [1-(2,3-dioleyloxy) propyl]-N,N,N-tri-methylammonium chloride (DOTMA) (Pinnaduwage et al. 1989). According to published results, neither the head group nor the tail(s) of the lipid are the determinant for cationic lipids being used as plasmid delivery vectors (Gao and Huang 1995, Rolland 1998). Therefore, it was hypothesized that with a suitable head group, single-tailed cationic lipids may function in gene delivery. We synthesized the single tail cationic

lipid oleyl ornithinate (OLON) (Fig.1) and used it for plasmid transfection in mammalian cells. To decrease the toxicity of the single tailed lipid, we also synthesized a single tail lipid-6-lauroxyhexyl ornithinate (LHON), with an ester bond in the tail (Figure 4-1). Cationic lipids with ester bonds are more biodegradable and therefore have been associated with less cytotoxicity (Farhood et al. 1992, Leventis and Silvius 1990). Recently, Nantz et al reported a novel tetraester construct that reduced cationic lipid-associated cytotoxicity (Aberle et al. 1998) using ester bonds as linkers to tether the head group and tail. Here, we address a strategy to introduce an ester bond into the tail. Whether the introduction of an ester bond to the single chain of LHON will have a detrimental effect on transfection activity is also addressed. The difference between LHON and its analog OLON is that LHON has an ester bond in the tail whereas OLON has a double bond in a similar position (oleyl, C18). The monounsaturated chain offers sufficient membrane fluidity and good lipid mixing within the bilayer at physiological temperature (Felgner et al. 1994, Felgner et al. 1987, Leventis and Silvius 1990). Due to flexibility of the ester bond, LHON may also have these properties. An additional ester bond in the tail further increases the chance of cationic lipid being degraded, thus decreasing the toxicity. We compared the transfections of double-tailed 1', 2'-dioleyl-sn-glycero-3'-succinyl-1, 6-hexanediol ornithine conjugate (DOGSHDO) which has an ornithine headgroup, single-tailed OLON which has an ornithine head group, double-tailed DOTAP which has a quaternary amine group, and single-tailed CTAB which has a quaternary amine group, in order to investigate the effect of single tailed lipids on transgene expression and cellular uptake of DNA/liposome complexes. At the optimal DNA/lipid ratios, OLON/DOPE had more than 10 times higher transgene expression than

the other three liposomes even though the DNA uptake was not higher. In the experiments involving the release of DNA from DNA/liposome complexes using an anionic substance, a greater fraction of DNA from DNA/OLON/DOPE complexes was released than that from DNA/DOTAP/DOPE complexes. The results indicated that single tailed compounds can function as DNA delivery vectors and the introduction of an ester bond within the alkyl chain can further reduce toxicity associated with cationic lipids.

Materials and methods

Chemicals

Lauric acid and oleyl alcohol were purchased from Fluka (Ronkonkoma, NY). DOTAP, DOPE, CTAB and N-(lissamine rhodamine B sulfonyl)-phosphatidylethenolamine (Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). DOGSHDO was synthesized according to a previous procedure (Tang and Hughes 1998). Acetoxymethyl ester of calcein (calcein-AM) was purchased from Molecular Probes, Inc. (Eugene, OR). 2-(*Tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON), triethylamine (TEA), L-ornithine hydrochloride, and 1,3-dicyclohexylcarbodiimide (DCC) were bought from Aldrich (Milwaukee, WI). Sodium dodecyl sulfate (SDS) and dextran sulfate (DS) were purchased from Sigma (St. Louis, MO).

Syntheses (Figure 4-2)

One equivalent of L-ornithine was stirred with 2.5 equivalents of BOC-ON in wet 1,4-dioxane at room temperature overnight to produce BOC-ornithine. One equivalent of BOC-ornithine was esterified with 5 equivalent of 1, 6-hexanediol to form 6-hydroxyhexyl BOC-ornithinate (DCC, 1.1 equiv., RT, in CH_2Cl_2) (Hassner and

Alexanian 1978). 6-hydroxyhexyl BOC-ornithinate and 1 equivalent of lauric acid were condensed to generate 6-lauroxyhexyl BOC-ornithinate (DCC, 1.1 equiv., RT, in CH_2Cl_2). Oleoyl BOC-ornithinate was synthesized by directly conjugating BOC-ornithine with oleyl alcohol (DCC, 1.1 equiv., RT, in CH_2Cl_2). All reactions were followed by thin layer chromatography (TLC). A normal silica gel chromatography method was used to purify the products. The overall yield was 60%-65% based on the initial amount of amino acid. The molecular weights were determined by a Micromass Quattro-LC-Z triple quadruple mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) ion source (Beverly, MA). 6-lauroxyhexyl BOC-ornithinate: MW=615.5 (calculated 615.8 for $C_{33}H_{62}N_2O_8$). ^1H NMR ($CDCl_3$, 300 MHZ): 0.89 [t, CH_3], 1.20-1.69 [m, 15×(CH_2)], 1.42 [s, 2×$(CH_3)_3CO$], 2.29 [t, $COCH_2$], 3.14 [q, CH_2N], 4.06 [t, CH_2OCO], 4.13 [t, CH_2OCO], 4.28 [s, α-H], 4.61 [broad s, NH], 5.03[d, NH]. Oleyl BOC-ornithinate: MW=583.5 (calculated 583.8 for $C_{33}H_{62}N_2O_6$). ^1H NMR ($CDCl_3$, 300 MHZ): 0.89 [t, CH_3], 1.20-1.69 [m, 14×(CH_2)], 1.42 [s, 2×$(CH_3)_3CO$], 2.01[m, $CH_2C{=}CCH_2$], 3.14 [q, CH_2N], 4.11 [t, CH_2OCO], 4.28 [s, α-H], 4.58 [broad s, NH], 5.03[d, NH], 5.30-5.41 [m, CH=CH]. Before preparation of liposomes, BOC groups were detached (CF_3COOH, 0^0C, 15 min), and excessive trifluoroacetic acid was dried under high vacuum to produce LHON and OLON. This step is quantitative as reported by Behr (Behr et al. 1989). The purity of the LHON and OLON were checked using TLC and then used to prepare liposomes without further purification.

Liposome Preparation

DOTAP, LHON, OLON, and DOGSHDO were dissolved in chloroform and mixed with a helper lipid DOPE (1:1 molar ratio). CTAB was mixed with DOPE using

an optimal molar ratio of 1:4 (Pinnaduwage et al. 1989). The mixture was evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The resulting lipid film was dried by nitrogen for ten additional minutes to evaporate any residual chloroform. The lipid film was suspended in sterile water to a concentration of 1 mg/ml based on the weight of the cationic lipids. The resultant mixtures were shaken in a water bath at 35 ^{0}C for 30 minutes. The suspensions were then sonicated using a Sonic Dismembrator (Fisher Scientific) for 1 minute at room temperature to form homogenized liposomes. The particle size distribution and zeta potential of liposomes were measured using a NICOMP 380 ZLS instrument (Santa Babara, CA). The diameters were expressed by the volume-weight distribution parameter. The average diameters of DOTAP/DOPE, LHON/DOPE and OLON/DOPE were 131.8 ± 17.3 nm, 141.0 ± 18.7 nm, and 173.6 ± 40.8 nm, respectively. The zeta potentials were + 9.02 mV, 4.08 mV, and 3.18 mV, respectively. Liposomes were stored at 4 ^{0}C and stable for at least 3 months without decreasing transfection activities (data not shown).

Plasmid DNA

All supplies were purchased from Promega (Madison, WI). Plasmid DNA was obtained from E. coli (strain JM-109) which had been transformed with pGL3, a luciferase producing plasmid with SV-40 promoter and enhancer sequences. Plasmid DNA was isolated using a Wizard Megaprep DNA purification kit from Promega. Plasmid DNA concentration and purity were determined using a spectrophotometer.

Gel Retardation Analysis of Binding of DNA/liposome Complexes

One μg of plasmid DNA (pDNA) was dissolved in 10 μl of phosphate-buffer saline (PBS). Cationic liposomes were added to a pDNA solution at the increasing

weight ratios from 0 to 6 respectively. All calculations of the weight of liposomes in this paper were based on the weights of the cationic lipids. The complexes were incubated at room temperature for 30 minutes and then analyzed on 1 % agarose gel. The amount of free DNA was analyzed using an electrophoresis documentation and analysis system (Kodak digital science, New York, NY).

Cell Culture and Luciferase Gene Transfection

Chinese hamster ovary (CHO) cells were obtained from American Tissue Type Collection (Rockville, MD). The cells were maintained in α-MEM media, and supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml). All cells were kept in 5% CO_2 and at 37 ^{0}C in humidified air. Cell lines were cultured and seeded in 24 well plates (2×10^5 cells/well) and grown to 60-80 % confluence in 1 ml of serum-containing media. DNA/liposome complexes were made and incubated at room temperature in serum free media for 30 minutes. Before transfection, the media was removed and cells were washed with PBS before the addition of 400 μl of serum free media to each well. DNA/liposome complexes in 100 μl of serum free media were added to each well and incubated for 4.5 hours. The transfection activities of single-tailed lipids OLON, CTAB, double-tailed DOGSHDO, and DOTAP were also compared. The weight ratios of DNA to lipid in DOTAP/DOPE, OLON/DOPE, DOGSHDO/DOPE, and CTAB/DOPE complexes were 1:2, 1:3, 1:8 (Tang et al. 1999), and 2:7 (Pinnaduwage et al. 1989), respectively. The optimal weight ratio of DNA to DOGSHDO/DOPE was based on the weight of DOGSHDO only, the optimal weight ratio of DNA to CTAB/DOPE was based on the weight of both CTAB and DOPE. After transfection for 4.5-hours, the transfection media were changed to

growth media and cells were incubated for another 24 hours. Luciferase activity was measured using a standard luciferase assay (Vigneron et al. 1996). Each experiment was repeated at least twice.

Flow Cytometry Analysis of the Cell Associated DNA/liposomes Complexes

A fluorescein labeling kit was purchased from PanVera Corporation (Madison, WI), and pDNA was labeled according to the product directions. Liposomes were labeled by incorporating 1 molar percent of Rh-PE in liposomes. One μg/well of fluorescein labeled pDNA was complexed with Rh-PE labeled DOGSHDO/DOPE, OLON/DOPE, DOTAP/DOPE, and CTAB/DOPE liposomes at the optimal DNA /liposome weight ratio of each liposome, respectively. The complexes were used for transfection as described previously with slight modifications. Serum free media at 4°C was used in the experiments involving transfection of the cells at 4 °C. After transfecting the cells for 4.5-hours at 37 °C or 4°C, the cells were quickly washed with PBS three times. The cells were incubated with 1 ml of CellSrub™ (washing) buffer (Gene Therapy System, San Diego, CA) for 10 minutes, to remove the complexes attached on the cell surface. After the cell washing buffer was removed and the cells were washed with PBS twice followed by 100 μl/well of 0.5 % of trypsin-EDTA. The cells were trypsinized for 2-3 minutes and the reaction was stopped by 200 μl of 1% of bovine serum albumin (BSA) in PBS. The cells were collected and centrifuged at 250 g for 10 minutes. The supernatant was decanted and the cell pellet was resuspended in 500 μl of 1% BSA in PBS and centrifuged for another 10 minutes to remove any remaining phenol red and extracellular liposome/DNA complexes. After the BSA solution was discharged, the cell pellet was resuspended in 500 μl of PBS and fixed in 500 μl of 2 %

paraformaldehyde in PBS. The cells were fixed at 0 ^{0}C for 15 minutes then stored at 4 ^{0}C overnight. Cell fluorescence was measured using a Becton Dickinson FACSort flow cytometer (San Jose, CA). 10,000 cells were counted for each treatment. Cells were gated by their morphological properties. The mean fluorescence intensity of the cells was measured in terms of arbitrary units using a LYSYS II software program (Becton Dickinson, San Jose, CA). The sample was excited at a wavelength of 488 nm. The fluorescence of fluorescein-DNA was measured at the wavelength of 530 nm (FL1) and the fluorescence of Rh-PE was measured at the wavelength of 580 nm (FL2). Cells transfected with non-labeled DNA and non-labeled liposome, with fluorescein-DNA and non-labeled liposome, and with non-labeled DNA and Rh-PE labeled liposomes, were used as controls. The background fluorescence intensity was subtracted. Each experiment was performed in triplicate and repeated twice.

Release of DNA from DNA/liposome Complexes by Sodium Dodecyl Sulfate (SDS) and Dextran Sulfate (DS)

Two μg of DNA was complexed with OLON/DOPE or DOTAP/DOPE at the -/+ charge ratio of 1:5 (12 μg of OLON/DOPE assuming one of primary amino group charged and 22 μg of DOTAP/DOPE) in 500 μl of PBS. At these concentrations, two complexes reached the same fluorescence intensity when exposed to SYBR Green I nucleic acid gel stain (Molecular Probe, Oregon). The complexes were incubated at room temperature for 30 minutes. Five hundred μl of 2× SYBR PBS solution was added and mixtures were incubated for another 15 minute. The fluorescence signal was measured at the excitation wavelength of 497 nm, and emission wavelength of 520 nm. The DNA to OLON/DOPE and DNA to DOTAP/DOPE complexes, initially had the same fluorescence intensity. To test the release of DNA by anionic compounds, DS and

SDS, increasing amounts of DS or SDS were added to complexes, and the resultant mixture was vortexed and the recovered fluorescence signal was measured. The release of DNA was expressed as fraction of fluorescence recovered

$$\text{Fraction of fluorescence recovered} = \frac{\text{Fluorescence intensity of sample/SYBR}}{\text{Fluorescence intensity of free DNA/SYBR}}$$

Toxicity Assay

The toxicity of LHON/DOPE, OLON/DOPE and DOTAP/DOPE in the CHO was determined using a CARE-LASS (a calcein release assay) (Lichtenfels et al. 1994). Before the assay, cells were plated in a 96 well plate (1×10^5cells/well) and grown to 60-80 % confluence overnight. To test the toxicity of different liposomes, increasing amounts if liposomes were added to cell cultures. To test the toxicity of the DNA/liposome complexes, a fixed amount of 1 μg/well of DNA was used to complex with increasing amount of liposomes. The ratios of DNA to liposome were the same as the ratios used in transfection studies. After specific treatments, cells were washed three times with PBS and incubated with 100 μl of calcein-AM (1 μg/ml in PBS) for 30 minutes at room temperature. Calcein fluorescence intensity was measured at the excitation and emission wavelengths of 485 nm and 538 nm using a Molecular Device f_{max} Plate Reader (Molecular Devices Corp., CA). The mean fluorescence intensity was calculated and compared to the mean fluorescence value for nontransfected cells. Both fluorescence values were subtracted by naturally released calcein fluorescence intensity. The final results were expressed in terms of percent cell survival. 100 % means no cytotoxicity and 0 % means no cells survived the treatments. Each experiment was performed in triplicate and repeated at least twice.

Statistical Analysis

Statistical analyses were were performed using ANOVA-Fisher's PLSD Post hoc tests (Stat View 4.53, Abacus Concepts, Inc., Berkeley, CA). One-way ANOVA was applied between different liposomes/DNA ratios of the same liposome. Two-way ANOVA was applied among treatments of liposome groups and liposomes/DNA ratios. A probability of less than 0.05 was considered to be statistically significant.

Results

Binding Assays

As shown in Figure 4-3, more than 99 % of DNA was complexed by the liposomes of LHON/DOPE and OLON/DOPE at the weight ratios from 1 to 6. When DNA was mixed with DOTAP/DOPE at a weight ratio of 1:1, 52% of DNA was free. At the ratio of 2:1, 92% of DNA was complexed.

Luciferase Plasmid Transfection

LHON/DOPE, OLON/DOPE, and DOTAP/DOTAP were used to deliver pGL3 luciferase plasmid to CHO cells. To screen the effects of DNA dose on the transgene expression, pDNA doses of 3 μg/well and 1 μg/well were evaluated. As shown in Figure 4-4A and 4-4B, LHON had similar transfection activity to OLON. The transfection activities of LHON/DOPE and OLON/DOPE peaked at weight ratio of 1:3. DOTAP/DOPE liposomes exhibited a maximum transfection activity at the weight ratio of 1:1 at DNA concentration of 3 μg/well (Figure 4-4A). It is worthy noting that, at the ratio of 1:1 only 48% of DNA was complexed although DOTAP/DOPE had greater transfection activity than at other ratio (Figure 4-3C). When 1 μg/well of DNA was used,

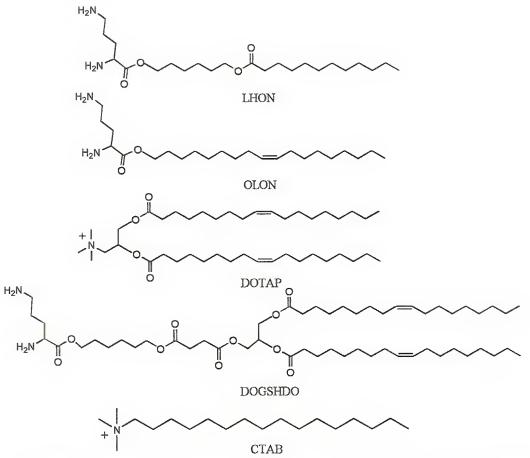


Figure 4-1: Chemical structures of 6-lauroxyhexyl ornithinate (LHON), oleoyl ornithinate (OLON), 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1', 2'-dioleyl-sn-glycero-3'-succinyl-1, 6-hexanediol ornithine conjugate (DOGSHDO), and cetyltrimethylammonium bromide (CTAB).

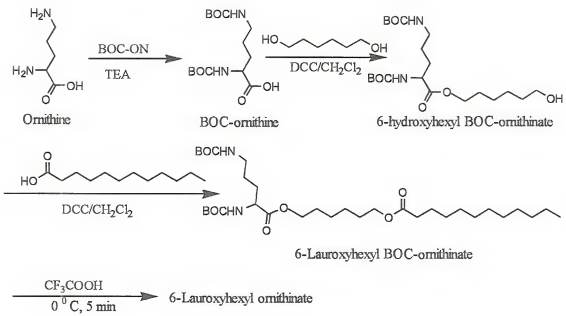


Figure 4-2: Schemes for the synthesis of 6-lauroxyhexyl ornithinate (LHON). BOC-ON,2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile; TEA, Triethylamine; DCC, 1,3-dicyclohexylcarbodiimide.

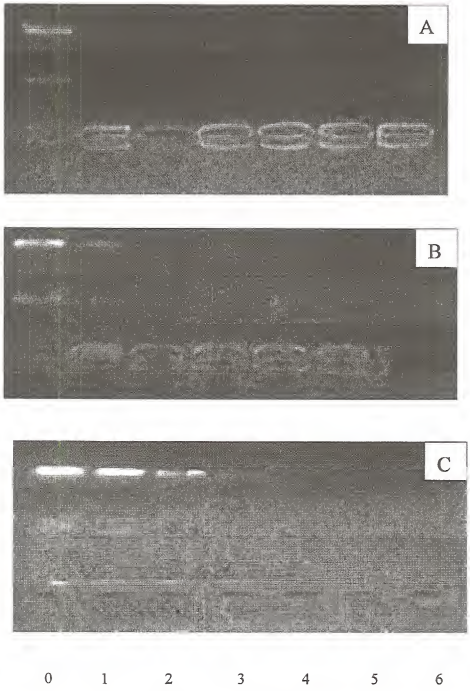


Figure 4-3: Gel retardation assays of DNA/liposome complexes. One μg of plasmid DNA was complexed with cationic liposomes LHON/DOPE, OLON/DOPE or DOTAP/DOPE at increasing weight ratios from 0 to 6 respectively. The complexes were analyzed by 1 % agarose gel electrophoresis as described in Methods. A: LHDO/DOPE/DNA, B: OLON/DOPE/DNA, and C: DOTAP/DOPE/DNA.

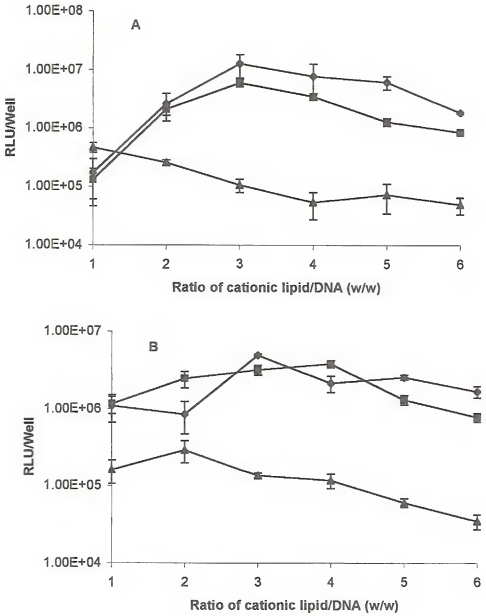


Figure 4-4: Comparison of the transfection of pGl3-luciferase plasmid by LHON/DOPE, OLON/DOPE and DOTAP/DOPE in CHO cells. A fixed dose of pGL3 DNA was mixed with increasing weight ratios of cationic liposomes (calculation based on cationic lipid) and used for transfection. A: 3 μg/well of pGL3 DNA, B: 1 μg/well of pGL3 DNA. ◆: LHON/DOPE, ■: OLON/DOPE; ▲: DOTAP/DOPE. Data is shown as mean ±S. D. (n=3).

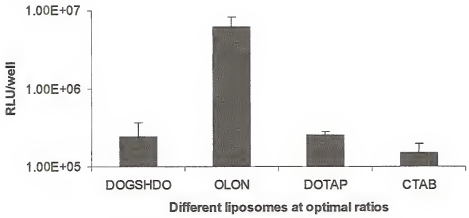


Figure 4-5: Comparison of the transfection of pGl3-luciferase plasmid by DOGSHDO/DOPE, OLON/DOPE, DOTAP/DOPE, CTAB/DOPE. 3 μg of pGl3 DNA was mixed with cationic liposomes DOGSHDO/DOPE, OLON/DOPE, DOTAP/DOPE, CTAB/DOPE at the optimal ratio of each liposome and used in transfection as stated in Methods. Transfection activity was expressed as RLU/well. Data is shown as mean ±S. D. (n=4).

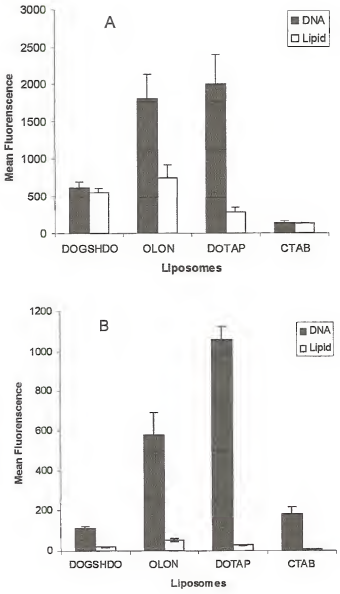
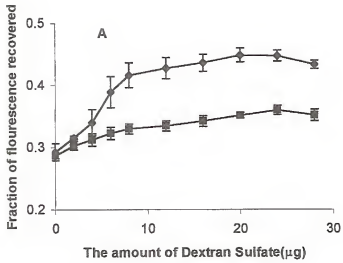


Figure 4-6: Comparison of uptake of DNA/liposome complexes by CHO cells. One μg/well of fluorescein labeled pDNA was complexed with Rh-PE-labeled DOGSHDO/DOPE, OLON/DOPE, DOTAP/DOPE, and CTAB/DOPE at the weight ratios stated in Fig. 5. After 4.5-hour transfection period, the fluorescence of fluorescein-DNA and Rh-PE was measured at different wavelength as indicated in Methods. A: incubation at 37 °C, B: incubation at 4 °C. Data is shown as mean ±S. D. (n=3).



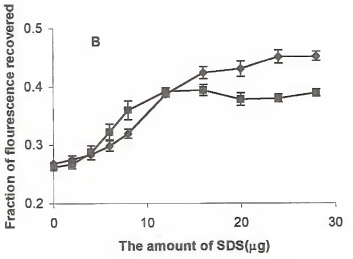


Figure 4-7: Release of DNA from DNA/liposome complexes by sodium dodecyl sulfate (SDS) and dextran sulfate (DS). Two μg of DNA was complexed with OLON/DOPE or DOTAP/DOPE at the -/+ charge ratio of 1:5 and mixed with SYBR. DS or SDS was added to complexes, and the resultant mixture was vortexed and recovered fluorescence signal of SYBR was measured as stated in Methods. ◆: OLON/DOPE, ■: DOTAP/DOPE. A: release by DS, B: release by SDS. Data is shown as mean ±S. D. (n=4).

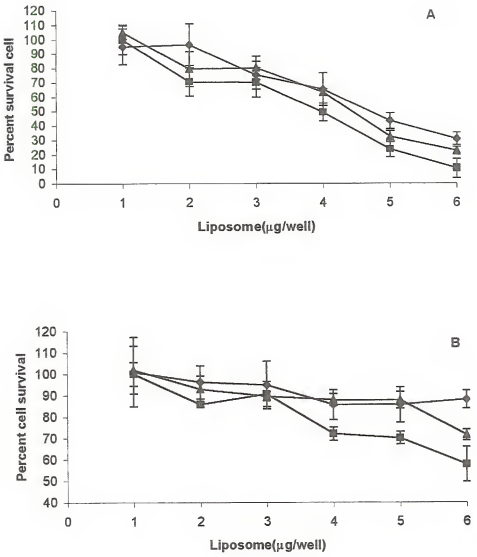


Figure 4-8: Toxicity assays. Cytotoxicity of liposomes and the liposome/DNA complexes in CHO cells. Liposomes only or a fixed dose of 1 μg/well of pDNA was mixed with increasing amount of cationic liposomes and used in the toxicity assay. Cell viability was calculated as percentage of survival cells as stated in Methods. A: liposomes only, B: DNA/liposome complexes. ◆: LHON/DOPE, ■: OLON/DOPE; ▲: DOTAP/DOPE. Data is shown as mean ±S. D. (n=3).

transfection activity of DOTAP/DOPE peaked at the ratio of 1:2; however, the transfection activity was not significantly different from DOTAP/DOPE at the weight ratio of 1:1 (Figure 4-4B, $p>0.05$). The optimal transgene expressions of LHON/DOPE an mmd OLON/DOPE liposomes were more than 10 times greater than that of DOTAP/DOPE liposomes (Figure 4-4) (similar transfection results were also demonstrated in COS 1 cells, data not shown). The DNA dose response was not linear for transgene expression. These results are in agreement with Farhood's (Farhood et al. 1992). As shown in Figure 4-4, DNA complexes of three times greater dose did not result in a corresponding increase in transgene expression. When 3 μg/well of DNA was used, the optimal transgene expression was only 1.5 to 2 times higher than the expression at a DNA concentration of 1 μg/well, instead of the expected 3 times. In order to compare the transfection efficiency of liposomes with the same head groups (i.e., ornithine or quaternary amine) but different tails (i.e., single tail or double tail), we compared the transfections of double tailed DOGSHDO which has an ornithine head group, single-tailed OLON which has a ornithine head group, double-tailed DOTAP which has a quaternary amine group, and single-tailed CTAB which has a quaternary amine group. As shown in Figure 4-5, OLON liposomes had more than 10 times higher transfection activity than other liposomes. CTAB/DOPE had a lower transfection activity than DOTAP/DOPE, although both lipids had a similar head group.

Cellular Associated DNA after Transfection Period

To investigate the possible reason why OLON/DOPE liposomes demonstrated the highest transfection activity, the cellular associated DNA/liposome complexes after transfection period in CHO cells at 37 ^{0}C was measured by flow cytometry (Figure 4-

6A). The relative amount of fluorescein-DNA and Rh-PE liposomes associated with cells was expressed by relative fluorescence intensity (Figure 4-6). The weight ratios of the lipids were the same as those used in the experiments which compared the different liposomes (Figure 4-5). The cellular associated fluorescence due to fluorescein-DNA delivered by DOGSHDO/DOPE and CTAB/DOPE was less than one-third that of both OLON/DOPE and DOTAP/DOPE (Figure 4-6A). It was interesting to note that the cellular uptakes of DNA complexed with either OLON/DOPE or DOTAP/DOPE were not significantly different, whereas their transgene expression was different by more than one order of magnitude (Figure 4-4, Figure 4-5). The weight ratios of DNA/liposome used in transfection of OLON/DOPE and DOTAP/DOPE were 1:3 and 1:2, respectively. The molar ratio of OLON/DOPE liposomes to DOTAP/DOPE liposomes ideally should be approximately 2.7, using the same concentration of DNA in both complexes. We found the ratio of uptake of OLON/DOPE to DOTAP/DOPE to be approximately 2.6, which agrees with this assumption (Figure 4-6A). To investigate the uptake mechanism, the transfection was also carried out at 4 (Figure 4-6B). As shown in Figure 4-6B, the degree to which cellular uptake decreased at 4°C from that measured at 37°C was not the same in all the treatments. At 4 °C the delivery of DNA by OLON/DOPE was shown to decrease by two-thirds and the delivery of DNA by DOTAP/DOPE decreased by one-half.

Release of DNA from DNA/liposome Complexes by Anionic Molecules

Although both OLON/DOPE liposomes and DOTAP/DOPE liposomes delivered the same amount of DNA to the cells, OLON/DOPE liposomes resulted in greater than 10 times higher transgene expression. The release of DNA from DNA/liposomes by

sodium dodecyl sulfate (SDS) or dextran sulfate (DS) was tested in order to investigate the reason for this phenomenon. As shown in Figure 4-7, DNA/OLON/DOPE complexes had about 45% of total fluorescence recovered by excess DS or SDS. DNA/DOTAP/DOPE complexes had less fluorescence recovery and only 35%-40% of fluorescence recovery in the presence of excessive DS or SDS. The recovery of fluorescence reached a plateau at the dose 12 μg of dextran sulfate (at the -/+ charge ratio of 2:1) and the further addition of DS or SDS did not help significant greater amount of DNA to be released (Figure 4-7A). When SDS was added to DNA/liposome complexes, the recovery of fluorescence of DNA/DOTAP/DOPE reached a plateau (12 μg of SDS (-/+ charge ratio of 1.3/1) earlier than that of DNA/OLON/DOPE (16 μg of SDS) (Figure 4-7B).

Toxicity Assay

The cytotoxicity of liposomes and DNA/liposome complexes was tested. In the complexes, 1 μg/well of DNA was mixed with liposomes with the same ratios as used in transfection experiments. As shown in Figure 4-8A, free liposomes were quite toxic to cells. At concentrations of 6 μg/well, only about 20 % cell survived. Without an ester bond, single tailed OLON liposomes had greater toxicity than DOTAP and LHON liposomes at 6 μg/well ($p<0.05$). As shown in Figure 4-8, DNA/liposome complexes had less toxicity than its free liposome formulation since DNA can cover part of toxicity by neutralizing the positive charges of liposomes. These results were consistent with that of Huang et al. (Gao and Huang 1991). In Figure 4-8B, at low liposome doses (1, 2, and 3 μg/well), the three liposomes did not have significant different cytotoxicity ($p>0.05$). At higher doses, OLON/DOPE showed greater toxicity than DOTAP/DOPE and

LHON/DOPE ($p < 0.05$) (Figure 4-8B). LHON/DOPE was also less toxic than DOTAP/DOPE at a dose of 6 μg/well (Fig.8B). LHON/DOPE showed lower toxicity than OLON/DOPE in COS 1 cells at high concentration (data not shown).

Discussion

In the current work, we presented a convenient method of synthesis of the single-tailed cationic lipid OLON and its analog LHON. The optimal transfection activities of LHON/DOPE and OLON/DOPE were more than one order of magnitude greater than that of DOTAP/DOPE (Figure 4-4). Although an ester bond linker has been used to decrease the toxicity of cationic lipids, the strategy of introducing an ester bond in the tail backbone has not previously been reported. LHON/DOPE showed similar transfection activities to its analog OLON/DOPE , therefore, the replacement of a double bond in the tail with an ester did not decrease the plasmid transfection activity of cationic lipid. It was worthy noting that the DOTAP/DOPE had optimal transfection activity at the cationic lipid/DNA ratio of 1:1 or 2:1 (Figure 4-4). The gel analysis showed that only 48% of total DNA was complexed at the ratio of 1:1(Figure 4-3). The results indicated that the percentage of DNA complexed by cationic liposomes is not the only determinate factor for transfection efficiency. The physical properties of the complexes may also play important roles in transfection.

As shown in Figure 4-5, liposomes composed of OLON had greater than 10 times transfection activity than its double-tailed analog DOGSHDO, whereas double-tailed DOTAP had higher transfection activity than its single-tailed analog CTAB. The results supported that neither the head group nor the tail group is the determinate of transfection activity of cationic lipids (Gao and Huang 1995, Rolland 1998). The quaternary amino

head group of DOTAP is always charged and strongly hydrophilic thus may require a lipophilic double chain tail group to counterpart it. The primary amino groups of ornithine are not as hydrophilic as quaternary amino group, so a single chain tail might be optimal to it.

To address a possible reason for the greater transfection activity of OLON/DOPE compared to DOGSHDO/DOPE, DOTAP/DOPE, and CTAB/DOPE, we used flow cytometry to analyze the delivery of fluorescein-DNA into cells. The application of flow cytometry methods to analyze the uptake of DNA/liposome complexes for plasmid delivery was previously reported by Zabner et al. (Zabner et al. 1995) and Tseng et al. (Tseng et al. 1997). The flow cytometry data of DNA uptake was consistent with data collected through DNA extraction methods (Legendre and Szoka 1992). It was not a surprise that both DOGSHDO and CTAB liposomes delivered less DNA into cells since DOGSHDO and CTAB liposomes had lower transgene expression than OLON/DOPE liposomes (Figure 4-5, Figure 4-6A). However, the delivery of DNA by OLON/DOPE was not significant different from that of DOTAP/DOPE ($p>0.05$) even though the transgene expression was different by one order of magnitude (Figure 4-6A). The phenomenon of achieving the same level of transgene expression after transfecting with lower amounts of DNA was previously reported by Huang et al (Yang and Huang 1998). According to Huang's observation, matured 3-β-[N-(N', N'-dimethylaminoethanol) carbarmoyl] cholesterol(DC-Chol)/DOPE/DNA complex delivered 10 times lower amounts of DNA to BL6 cells in the presence of serum than in the absence of serum whereas the transgene expression levels were same. Huang rationalized that some components of serum may have played a dominant role in DNA trafficking to the nucleus

from the cytoplasm. Since transfections in our experiments were carried out in the serum free conditions, other factors, such as release of DNA from complexes and escape of the complexes from endosomes, could play a role.

The release of DNA from endosomes may play a key role in the enhancement of transgene expression. Several groups have suggested that a major barrier to cationic liposome-mediated gene transfection is the ability of the endocytosed liposome/DNA complexes to destabilize the endosome membrane and release plasmid DNA into cytoplasm (Escriou et al. 1998a, Rolland 1998, Zabner et al. 1995). LHON and OLON have two primary amino groups in the ornithine head group. The pKa of the primary amino group are similar to those of the tertiary amino group since most alkaylamines have pKas in the narrow range of 10-11, regardless of their substitution pattern (McMurry 1988). Barenholz and Zuidam determined electrostatic parameters of DC-Chol/DOPE and DOTAP/DOPE and their DNA complexes (Zuidam and Barenholz 1997, Zuidam and Barenholz 1998). According to their results, at pH of 7.4, DC-Chol/DOPE was only 50 % charged and DOTAP/DOPE was 100 % charged. These results may also be applicable to the LHON/DOPE and OLON/DOPE. After cationic liposomes deliver plasmid DNA into cells by endocytosis, the endosome pH will decrease, resulting in a portion of the uncharged primary amino groups of the ornithine heads on LHON or OLON becoming charged. The increase of charge density of complexes has two effects. First, the buffer effect will slow down the decrease of pH in the endosomes. Secondly, the increase of charge density will increase the ability of DNA/liposome complexes to destabilize the endosome membrane thus helping the complexes escape from endosomes. Since DOTAP/DOPE liposomes are 100 % charged, the change of pH in cells will not

have an effect on the escape of complexes from the endosomes. DNA must also be released from complexes to allow transcription (Zabner et al. 1995). The release of DNA may occur in either the endosomes (Xu and Szoka 1996) or the cytoplasm after the complexes escape from the endosomes. The interaction between cationic liposome and DNA is a determinant factor. A strong interaction will decrease the release of DNA from complexes. DOTAP/DOPE delivered more DNA to cells at the weight ratio of 3/1 than at 1/1 (data not shown), whereas the transfection at 1/1 was greater (Figure 4-4). With a greater fraction of cationic DOTAP in the complexes, it may be more difficult for DNA to dissociate from the complexes. Another possibility is that a larger amount of cationic liposome in the complexes may neutralize part of the anionic macromolecules, which help DNA to escape from endosomes (Bhattacharya and Mandal 1998). According to Barenhoz, the different protonation of DC-Chol/DOPE and DOTAP/DOPE might result in a different distance between the headgroup and the phosphate group of plasmid DNA (Zuidam and Barenholz 1998). It is likely that dissociation of DNA from LHON/DOPE or OLON/DOPE requires less energy than fully charged DOTAP/DOPE thus resulting in greater amounts of release of DNA from complexes.

Cytoxicity is a major shortcoming of cationic-mediated gene transfection. A potentially biodegradable ester bond is usually used to tether the headgroup and tails to decrease the toxicity. Theoretically, the ester bond is not stable in aqueous solution. However, the chance of the ester bond being exposed to water is greatly decreased when cationic lipids are formulated as liposomes. Some liposomes with ester bonds are fairly stable and can be stored at 4 ^{0}C for months (Lasic 1997). The toxicity of free cationic liposomes can be partially be masked by DNA (Gao and Huang 1991). After DNA is

dissociated from the complexes, free cationic lipids may fuse with cell membranes thus causing membrane rupture. Single-tailed cationic lipids have been reported to be more toxic than their double-tailed counterpart (Pinnaduwage et al. 1989). Lasch et al. proposed that a single-tail detergent inserted into the bilayer membrane would result in regions of local curvature leading to membrane rupture (Lasch 1995). As shown in Figure 8, single-tailed OLON liposomes indeed had greater toxicity than those of DOTAP at high concentrations in CHO cells. However, with a potentially biodegradable ester bond in the tail, LHON had less toxicity than OLON and DOTAP (Fig. 8).

In summary, the single-tailed liposome OLON/DOPE demonstrated greater gene transfection activity than DOTAP/DOPE in CHO cells. Introduction of an ester bond into LHON decreased the toxicity of cationic lipid without sacrificing the transfection activities. OLON/DOPE liposomes achieved a greater transgene expression without a greater gene delivery than DOTAP/DOPE. The results may explained by the previous hypothesis that the escape of DNA from endosomes and release of DNA from complexes may be a major barrier of cationic lipid-mediated gene transfection (Escriou et al. 1998b, Rolland 1998, Zabner et al. 1995).

CHAPTER 5
INVESTIGATION OF THE HELPER FUNCTIONS OF 1, 2-DIOLEOYL-SN-GLYCERO-3-PHOSPHOCHOLINE (DOPC) IN LIPOSOME-MEDIATED PLASMID DNA DELIVERY

Introduction

Cationic liposomes are popular gene delivery vectors for *in vitro* and *in vivo* studies (Felgner et al. 1995, Gao and Huang 1995, Lee and Huang 1997, Rolland 1998). Most cationic liposomes containing a helper lipid-1, 2-dioleoyl phosphoethanolamine (DOPE) work more efficiently than formulations without DOPE *in vitro* (Farhood et al. 1995, Felgner et al. 1995, Gao and Huang 1991, Lee et al. 1996, Tang and Hughes 1998, Xu and Szoka 1996, Yang and Huang 1998). The replacement of DOPE with another neutral lipid-1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), which has the same backbone as DOPE but a choline headgroup instead of ethanolamine (Figure 5-1), was reported to reduce transfection activities of cationic liposomes (Farhood et al. 1992, Farhood et al. 1995, Felgner et al. 1994, Legendre and Szoka 1992, Zhou and Huang 1994). The helper function of DOPE was attributed to its fusogenic properties (Xu and Szoka 1996, Zhou and Huang 1994). It was rationalized that the propensity of DOPE to form a hexagonal structure helps endocytosed DNA/liposome complexes to destabilize (Xu and Szoka 1996, Zhou and Huang 1994). DOPE was also reported to be a helper lipid *in vivo* for lung delivery by nasal instillation (Lee et al. 1996). However, when liposomes were used to deliver plasmid DNA intravenously, liposomes without DOPE were reported to be more efficient than liposomes containing DOPE as a helper lipid (Liu

et al. 1999b). No mechanism was postulated to explain the difference. We have previously synthesized cationic lipids containing disulfide bonds and achieved effective transgene expression *in vitro* (Tang and Hughes 1998, Tang and Hughes 1999). DOPE demonstrated helper functions for these cationic lipids. In moving disulfide lipids to *in vivo* studies, we designed and synthesized quaternary ammonium containing structures since this permanent charge on the lipid may enhance activity *in vivo*. Based the success of the cholesterol backbone the lipid N', N', N'-trimethylaminoethyl-1'-cholesteryl-3, 3'-dithiodipropionamide (QS3) was made. In order to compare the effects of disulfide linker, we also synthesized its non-disulfide lipid analog-N', N', N'-trimethylaminoethyl-1'-cholesteryl-3, 3'-dithiodipropionamide (QN3) (Fig.1). In the normal formulating and testing of these compounds *in vitro*, we were surprised to observe that liposomes containing DOPC demonstrated greater transfection activities in CHO cells than those containing DOPE. The purpose of this study is to present a new method of synthesis of disulfide cationic lipids with a quaternary amine head group, to address why DOPC may also be a helper lipid in some formulations or in some cell types, and to present the results of a series of experiments which were conducted to address the mechanistic details involved in gene delivery with these new lipids.

Materials and Methods

DOPC and DOPE were purchased from Avanti Polar Lipids (Alabaster, AL). Acetoxymethyl ester of calcein (calcein-AM) was purchased from Molecular Probes, Inc. (Eugene, OR). 3-β-[N-(N', N'-dimethylaminoethanol) carbarmoyl] cholesterol (DC-Chol) was purchased from Sigma Biochemicals (St. Louis, MO). Other chemicals were

bought from Aldrich (Milwaukee, WI). pGL3-Luciferase control plasmid was prepared as previously reported (Tang and Hughes 1999).

Synthesis of QS3 and QN3

A total of 1.35 g of 3, 3'-dithiodipropinonic acid (6.4 mmol) and 1.00 g of cholesterol (2.5 mmol) were dissolved in 30 ml of N, N-dimethylacetamide. 0.1 ml of triethylamine and 2.5 mmol of 1,3 - dicyclohexylcarbodiimide (DCC) were added to the solution. The reaction mixture was stirred under nitrogen at room temperate overnight. Two ml of acetic acid was added to mixture and then the mixture was filtered and washed by 50 ml of water. The product was extracted with 50 ml of chloroform. The organic layer was washed four times with brine and dried over anhydrous sodium sulfate. The solvent was evaporated. The product, cholesteryl hemi-3, 3'-dithiodipropionate, was purified on silica gel with a developer composed of hexane: ethylacetate (4:1). A total of 0.80 g of cholesteryl hemi-3, 3'-dithiodipropionate (1.4 mmol) and 0.15 ml of N', N'-dimethylethylamine (1.4 mmol) were dissolved in 30 ml of methylene chloride and followed by 1.5 mmol of DCC. The reaction mixture was stirred at room temperature under nitrogen overnight. The reaction mixture was filtered and the solvent was evaporated. The product N', N'-dimethylaminoethyl-1'-cholesteryl-3, 3'-dithiodipropionamide was purified on silica gel with a developer of methylene chloride: methanol at the ratio of 10:1. Finally, 0.2 g of N', N'-dimethylaminoethyl-1'-cholesteryl-3, 3'-dithiodipropionamide (0.3 mmol) and 0.043 ml of dimethylsulfate (0.45 mmol) was dissolved in 5 ml of methylene chloride and stirred under nitrogen at room temperature overnight. The product QS3 (Fig.1) was crystallized three times from methylene chloride by ether. The overall yield was 30 %. The molecular weight was determined by a

Micromass Quattro-LC-Z triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) ion source (Beverly, MA). M = 665.13 (calculated, 665.10 for $C_{38}H_{67}N_2O_3S_2$). ^{1}H NMR ($CDCl_3$, 300MHZ): 0.70 (s, CH_3), 0.82-0.88 (dd, 2×CH_3), 0.84-2.04 [m, 2×(CH_3), 10×(CH_2), and 6×(CH)], 2.28-2.36 (d, CH_2), 2.62-2.74 (m, 2×CH_2), 2.88 –2.98 (m, 2×CH_2), 3.24-3.30 (s, 3× CH_3), 3.72 (b, 2×CH_2), 4.68 (m, CHO), 5.38 (m, C=CH), 8.0 (broad, NH). The same method was used to synthesize its non-disulfide analog N', N', N'-trimethylaminoethyl-1'-cholesteryl-3, 3'-dithiodipropionamide (QN3) and the product was checked by Mass and NMR spectrum.

Liposome Preparation and Particle Size Measurement

Lipids were dissolved in chloroform with or without a neutral helper lipid DOPE or DOPC (1:1 molar ratio). DC-Chol/DOPE was prepared by dissolving DC-Chol in chloroform and mixed with DOPE at the optimal molar ratio of 6:4 according to Gao and Huang (Gao and Huang 1991). The mixtures were evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The lipid film was dried by nitrogen for a further ten minutes to evaporate residual chloroform and the lipid film was resuspended in sterile water to a concentration of 1 mg/ml based on the weight of cationic lipids. The resultant mixtures were shaken in a water bath at 35 ^{0}C for 15 minutes. The liposomes were hydrated at 4 ^{0}C overnight. The suspensions were sonicated using a Sonic Dismembrator (Fisher Scientific) for 20 second with the output energy of 5 Watts at 4 ^{0}C to form homogenized liposomes. The particle size distributions of liposomes were measured using a NICOMP 380 ZLS instrument (Santa Babara, CA). The diameters were expressed by the volume-weight distribution parameter. To measure

particle sizes of liposome/DNA complexes, the preparation of liposome/DNA complexes mimicked the preparation liposome/DNA complexes used for transfection. Two μg of pGL3-DNA was dissolved in 100 μl of serum free α-MEM media and mixed with increasing amounts of different formulations. The mixtures were incubated at room temperature for 15 minutes before the particle sizes were measured.

Cell Culture and Luciferase Gene Transfection

Chinese hamster ovary (CHO) cells and human neuroblastoma SKnSH cells were obtained from American Tissue Type Collection (Rockville, MD). The cells were maintained in α-MEM or RPMI growth media, respectively. Cells were kept in 5 % CO_2 and at 37 ^{0}C in humidified air. Cells of same passage from the same flask were cultured and seeded in three 24 well plates (1×10^5 cells/well) and grown overnight in 1 ml of serum-containing media. pGL3 luciferase DNA/liposome complexes were made and incubated at room temperature in serum free media for 15 minutes and used for transfection. Before transfection, growth media containing serum was changed to 500 μl serum free media. DNA/liposome complexes in 100 μl of serum free media were added to each well and incubated for 4.5 hours. The comparison experiments testing the effects of chloroquine or cytochalasin B were conducted side by side. Complexes of DNA/liposomes at a weight ratio of 1:3 were added to neighboring columns. Serum free media was added to one set while serum free media containing 100 μM chloroquine or 20 μg/ml of cytochalasin B was added to the other set. After the 4.5-hour transfection period, the transfection media was changed to growth media without inhibitors and cells were incubated for another 44 hours. Luciferase activity was measured using a standard luciferase assay (Tang and Hughes 1998). Luciferase activity in transfected cells was

expressed as relative light unit (RLU) per well. All conditions for comparison of transfection activities of different kinds of liposomes were strictly maintained (Tang and Hughes 1999). Experiments were done in triplicate within a given series. The experiments were repeated at least twice.

Toxicity Assay

The toxicity of DNA/cationic liposomes was determined using a CARE-LASS (a calcein release assay) (Lichtenfels et al. 1994). Before the assay, cells were plated in a 96 well plate (2.5×10^4 cells/well) and grown overnight. To test toxicity increasing amounts of liposomes were added to cell cultures. To test the toxicity of the DNA/liposome complexes, a fixed amount of DNA of 0.5 μg/well was used to complex with increasing amount of liposomes. The ratios of DNA to liposome were the same as the ratios used in transfection studies. After specific treatments, cells were washed three times with PBS and incubated with 100 μl of calcein-AM (1 μg/ml in PBS) for 30 minutes at room temperature. Calcein fluorescence intensity was measured at the excitation and emission wavelengths of 485 nm and 538 nm using a Molecular Device f_{max} Plate Reader (Molecular Devices Corp., CA). The mean fluorescence intensity was calculated and compared to the mean fluorescence value for nontransfected cells. Both fluorescence values were subtracted by naturally released calcein fluorescence intensity. The final results were expressed in terms of percent cell survival. 100 % means no cytotoxicity and 0 % means no cells survived the treatments. Each experiment was performed in triplicate and repeated at least twice.

Release of DNA from DNA/liposome Complexes by Dextran Sulfate Sodium (DS)

Two μg of pGL3-DNA was complexed with different liposomes at the weight ratio of DNA/catonic lipid of 1:3 in 500 μl of PBS. The complexes were incubated at room temperature for 30 minutes. Five hundred μl of 2× SYBR Green I nucleic acid gel stain (Molecular Probe, Oregon) phosphate buffered solution was added and mixtures were incubated for another 15 minute. The fluorescence signal was measured at the excitation wavelength of 497 nm, and emission wavelength of 520 nm on a Perkin Elmer LS 50B Luminescence Spectrometer (Perkin Elmer Limited, UK). To test the release of DNA by anionic compounds, increasing amounts of DS were added to complexes, the resultant mixture was vortexed, and the recovered fluorescence signal was measured. The release of DNA was expressed as percent of fluorescence recovered.

Flow Cytometric Analysis of the Cellular Associated DNA

pGL3-DNA was labeled using a fluorescein labeling kit purchased from PanVera Corporation (Madison, WI). Two μg/well of fluorescein labeled pDNA was complexed with liposomes at the DNA /liposome weight ratio of 1:3. The complexes were used for transfection as described previously. After transfecting the cells for 4.5-hours at 37 °C, the cells were quickly washed three times with PBS. The cells were incubated with 1 ml of CellSrub™ (washing) buffer (Gene Therapy System, San Diego, CA) for 10 minutes, to remove the complexes attached on the cell surface. After the cell washing buffer was removed and the cells were washed with PBS twice followed by 100 μl/well of 0.5 % of trypsin-EDTA. The cells were trypsinized for 2-3 minutes and the reaction was stopped by 200 μl of 1% of bovine serum albumin (BSA) in PBS. The cells were collected and centrifuged at 250 g for 10 minutes. The supernatant was decanted and the cell pellet

was resuspended in 500 µl of 1% BSA in PBS and centrifuged for another 10 minutes to remove any remaining phenol red and extracellular liposome/DNA complexes. After the BSA solution was discarded, the cell pellet was resuspended in 500 µL of PBS and fixed in 500 µl of 2 % paraformaldehyde in PBS. The cells were fixed at 0 ^{0}C for 15 minutes then stored at 4 ^{0}C overnight. Cell fluorescence was measured using a Becton Dickinson FACSort flow cytometer (San Jose, CA). 10,000 cells were counted for each treatment. Cells were gated by their morphological properties. The mean fluorescence intensity of the cells was measured in terms of arbitrary units using a LYSYS II software program (Becton Dickinson, San Jose, CA). The sample was excited at a wavelength of 488 nm. The fluorescence of fluorescein-DNA was measured at the wavelength of 530 nm. Cells transfected with non-labeled DNA, with fluorescein-DNA and non-labeled liposomes were used as controls. The background fluorescence intensity was subtracted. Each experiment was performed in triplicate.

Electron Microscopic Analysis the Structure of DNA/liposome Complexes

Samples were prepared by complexing pDNA (2 µg/100 µl) and liposomes (6 µg/100 µl) in α-MEM serum free media and the complexes were incubated for 15 min at room temperature. The structures were visualized by electron microscopy. Carbon films, made by evaporation onto freshly cleaved mica, were placed on 400 mesh nickel grids. Suspensions of DNA/liposome complexes were adsorbed to the surface of the films and the grids were exposed to 1 % aq OsO_4 for 1 hour, washed with water and air dried. Specimens were rotary shadowed with carbon/platinum by electron beam evaporation at a 10 ^{0} angle and observed at 75 kV on a Hitachi H-7000 transmission electron microscope.

Statistical Analysis

Statistical analyses were performed using ANOVA-Fisher's PLSD Post hoc tests (Stat View 4.53, Abacus Concepts, Inc., Berkeley, CA). One-way ANOVA was applied between different liposomes/DNA ratios of the same liposome. Two-way ANOVA was applied among treatments of liposome groups and liposomes/DNA ratios. A probability of less than 0.05 was considered to be statistically significant.

Results

Transfections in CHO Cells

As shown in Figure 5-2, the formulations of DC-Chol/DOPC, QS3/DOPC, and QN3/DOPC had greater transfection activities than the formulations composed of cationic lipids only or cationic lipid/DOPE at their optimal ratios ($p < 0.05$). The transfection activity of DC-Chol/DOPC at the ratio of DNA/cationic lipid of 1:4 was about 4.5 times greater than that of DC-Chol/DOPE liposome at the optimal ratio of 1:2 (Figure 5-2A). The optimal transfection activity of DC-Chol/DOPC was also 8-fold greater than DC-Chol liposome without a helper lipid (Figure 5-2A). In the QS3 series, QS3 liposomes formulated with the helper lipid DOPC (at ratio of 1:4) showed 6-fold greater activity than QS3/DOPE (at the ratio of 1:6) and 20 times greater transfection activity than QS3 only (at the ratio of 1:6) (Figure 5-2B) ($p<0.05$). As shown in Figure 5-2C, at the ratio of 1:3, QN3/DOPC had 1.4 fold greater transfection activity than QN3 at the ratio of (1:5) and about 40 times greater than that of QN3/DOPE ($p<0.05$). Overall, at optimal ratio, disulfide liposomes QS3/DOPC had about two-folder greater activity than DC-Chol/DOPC and 1.4-folder greater activity than its analog QN3/DOPC (Figure 5-2) in CHO cells.

Transfection in SKnSH Cells

To investigate whether the effects of DOPC are cell dependent, the transfection activities of the liposome formulations were tested in SKnSH cells. The weight ratios of DNA to lipid of 1:2 and 1:4 were used since our preliminary data showed these two ratios could represent the transfection activity of each lipid in SKnSH cells. As shown in Figure 5-3, the results were different from those in CHO cells. All three formulations composed of the helper lipid DOPE had greater activities than cationic liposomes with or without the helper lipid DOPC ($p<0.05$). These results were in agreement with most published data (i.e. DOPE is a better helper lipid than DOPC). At the optimal ratio of DNA/cationic lipid of each liposome, DC-Chol/DOPE had 20-times greater transfection activity than QS3/DOPE and 100-folder greater activity than QN3/DOPE ($p<0.05$).

The Effects of Helper Lipids on Toxicity

In order to determine whether the enhancement of transgene expression by DOPC formulations in CHO cells resulted from differences in toxicity, we tested the toxicity of each formulation in CHO cells. As shown in Figure 5-4A, DOPE increased the toxicity of DC-Chol lipid ($p<0.05$). The toxicity of complexes of DNA/DC-Chol/DOPE was greater than that of DNA/DC-Chol at the ratios of DNA/cationic lipid of 1:2 to 1:6 ($p<0.05$). However, DC-Chol/DOPC had less toxicity than DC-Chol/DOPE. Although the trend of toxicity of DC-Chol/DOPC was slightly greater than that of DC-Chol, DC-Chol/DOPC showed significant difference only at the ratio of 1:5 ($p<0.05$). The presence of DOPE in cationic liposomes has previously been reported to increase cytotoxicity (Filion and Phillips 1997). The toxicity of QS3 and QN3 were less than that of DC-Chol (Figure 5-4B, 5-4C). DOPE did not increase the toxicity of QS3 or QN3 ($p>0.05$). At

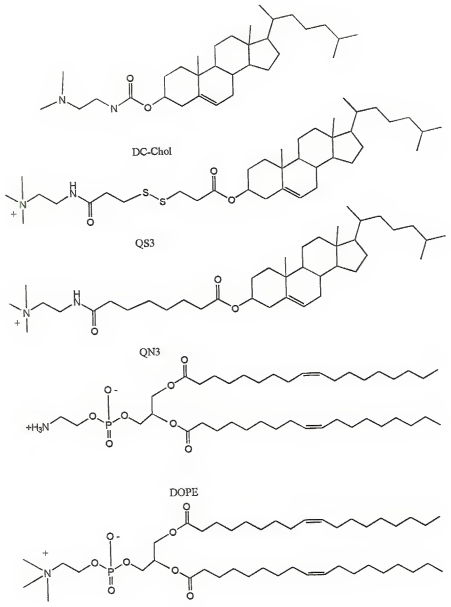


Figure 5-1: Structures of cholesteryl N-(dimethylaminoethyl)carbamate (DC-Chol), N', N', N'-trimethylaminoethyl-1'-cholesteryl-3, 3'-dithiodipropionamide (QS3), N', N', N'-trimethylaminoethyl-1'-cholesteryl-3, 3'-dithiodipropionamide (QN3), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).

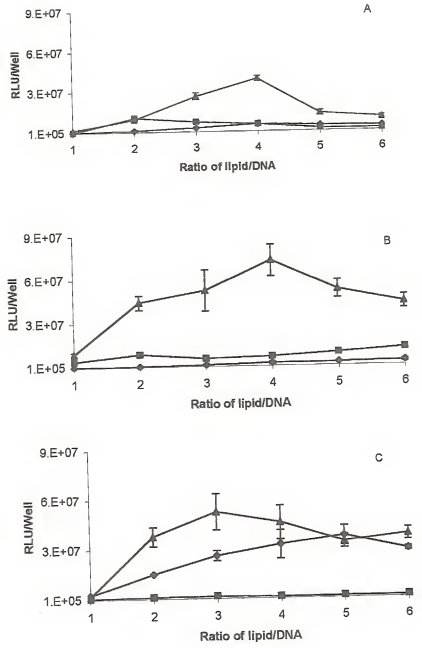


Figure 5-2: Comparison of the transfection activities of liposome formulations in CHO cells. A fixed dose of 2 μg/well of pGL3 DNA was mixed with increasing weight ratios of cationic liposomes (calculation based on cationic lipid) and used for transfection. A: DC-Chol series, B: QS3 series, C: QN3 series,◆: DC-Chol only, QS3 only, or QN3 only, ■: DC-Chol/DOPE; QS3 /DOPE, or QN3/DOPE, ▲: DC-Chol/DOPC, QS3/DOPC, QN3/DOPC. Data are shown as mean ±S. D. (n=3).

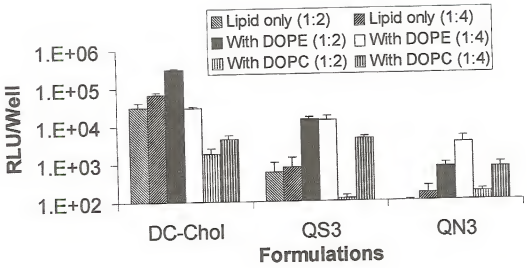


Figure 5-3: Comparison of the transfection activities of liposome formulations in SKnSH cells. A fixed dose of 2 μg/well of pGL3 DNA was mixed with cationic liposomes at the weight ratios of 1:2 or 1:4 (calculation based on cationic lipids) and used for transfection as stated in Methods. Data are shown as mean ±S. D. (n=3).

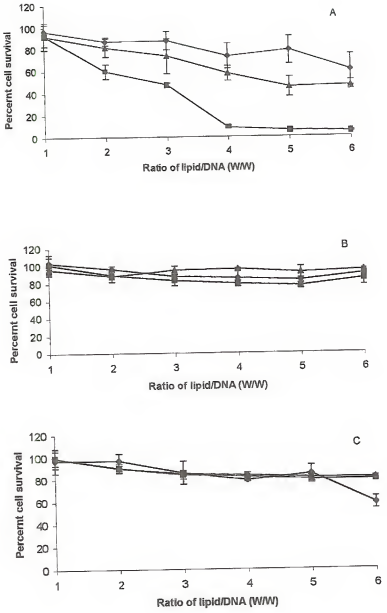
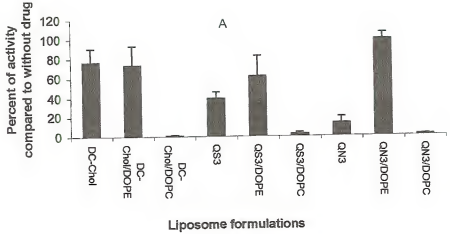


Figure 5-4: Comparison of cytotoxicity of DNA/liposome complexes of liposome formulations in CHO cells. A fixed dose of DNA was mixed with increasing weight ratios of cationic liposomes (calculation based on cationic lipid) and used for toxicity assay as in Methods. A: DC-Chol series, B: QS3 series, C: QN3 series,◆: DC-Chol only, QS3 only, or QN3 only, ■: DC-Chol/DOPE; QS3 /DOPE, or QN3/DOPE , ▲: DC-Chol/DOPC, QS3/DOPC, QN3/DOPC. Data are shown as mean ±S. D. (n=3).



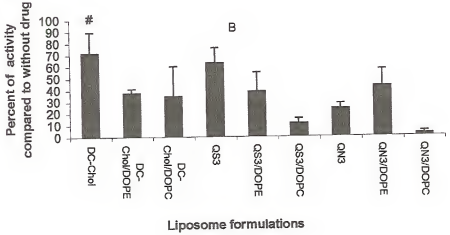


Figure 5-5: The effects of chloroquine or cytochalasin B on transfection activities of liposome formulations. The transfection activity in the presence of 100 μM chloroquine compared to in the absence of chloroquine. B: In the presence of 20 μg/ml of cytochalasin B. Experiments were done according to Methods. The data were normalized to percent of remained activities compared to normal transfection activities without chemical treatments. Data are shown as mean ±S. D. (n=3). #: The real value of DC-Chol formulation was divided by 3 to fit the axis range (its transfection activity was increased by cytochalasin B).

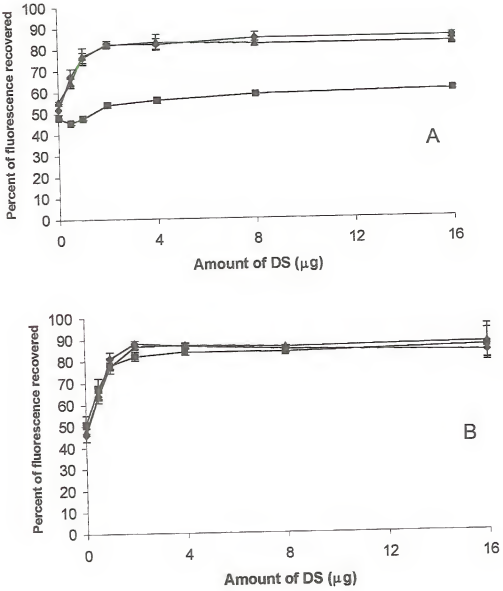


Figure 5-6: Release of DNA from DNA/liposome complexes by dextran sulfate (DS). Two μg of DNA was complexed with different lipsomes at the weight ratio of 1:3 and mixed with SYBR nucleic acid stain. DS was added to complexes, and the resultant mixture was vortexed and recovered fluorescence signal of SYBR was measured as stated in Methods. A: DC-Chol series, B: QS3 series. ◆: DC-Chol only, or QS3 only, ■: DC-Chol/DOPE; or QS3 /DOPE, ▲: DC-Chol/DOPC, or QS3/DOPC. Data are shown as mean ±S. D. (n=4).

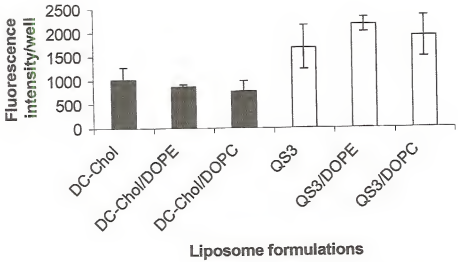


Figure 5-7: Comparison of the cellular associated pDNA transfected by liposome formulations in CHO cells. Two μg/well of fluorescein labeled pDNA was complexed with DC-Chol or QS3 liposomes at the weight ratio of cationic lipid to DNA of 3:1, respectively. The relative amount of pDNA was measured as relative mean fluorescence intensity of cells as indicated in Methods. Data are shown as mean ±S. D. (n=3).

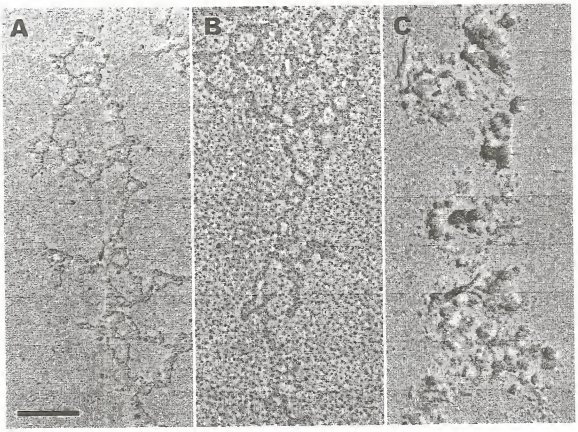


Figure 5-8: Structures of the major population of complexes of DNA/liposomes at the ratio of 1:3. The complexes were made in serum free media mimicking transfection conditions. The structures were analyzed as stated in Methods. A: DNA/DC-Chol, B: DNA/DC-Chol/DOPE, C: DNA/DC-Chol/DOPC. Bar = 0.5 μm.

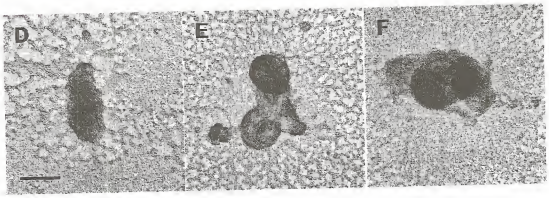


Figure 5-9: Structures of the major population of complexes of DNA/liposomes at the ratio of 1:3. The complexes were made in serum free media mimicking transfection conditions. The structures were analyzed as stated in Methods. A: DNA/QS3, B: DNA/QS3/DOPE, C: DNA/QS3/DOPC. Bar = 1.0 μm.

the ratio of 1:6, complexes of DNA/QN3 liposomes showed significant toxicity ($p<0.05$) (Figure 5-4C).

The Effects of Chloroquine or Cytochalasin B on Transfection Activities

As shown in Figure 5-5A, the transfection activity of each formulation was inhibited to some degree in the presence of 100 μM chloroquine. The transfection activities of DOPC formulations were dramatically decreased. The activities of DC-Chol/DOPC, QS3/DOPC, and QN3/DOPC were decreased to less than 5 % of the activity in the absence of chloroquine. Formulations composed of DOPE were less affected in all three cationic lipids. In the presence of 20 μg/ml of cytochalasin B, the transfection activity of DC-Chol liposomes was enhanced 2 fold. Similar enhancement was previously reported by Legendre and Szoka (Legendre and Szoka 1992), and the mechanism was not understood. Transfection activities of other formulations were decreased to a range of 80 % (QS3) to 5 % (QN3/DOPC) of the activities in normal serum free media without cytochalasin B. The transfection activity of QS3/DOPC and QN3/DOPC was greatly affected and decreased to less than 15 % of the control activity.

Release of DNA from DNA/liposomes Complexes

To investigate whether the incorporation of a helper lipid in cationic liposomes alters the interactions of DNA /cationic liposme, an anionic macromolecule dextran sulfate sodium salt (DS) was used to release DNA from the complexes. It was interesting to note that DOPE increased the stability of complexes of DNA/DC-Chol/DOPE (Figure 5-6A), however, it did not change the complexation in QS3 formulations (Figure 5-6B). When about 85 % of fluorescence was recovered from all DNA/liposome complexes except DNA/DC-Chol/DOPE, the release of DNA by DS reached a plateau (Figure 5-6).

In the presence of excess anionic DS, only about 55 % of fluorescence was recovered from complexes of DNA/DC-Chol/DOPE. Prolonged incubation period in the presence of DS did not increase the release of DNA from complexes. After the complexes were incubated overnight in the presence of excess dextran sulfate (i.e., 16 μg/ml, charge ratio of -(DS)/+(cationic lipid)=16/1) at room temperature, the maximum value of release did not increase for any one of the six complexes (data not shown).

Cellular Associated DNA

The amount of cellular associated fluorescein-labeled DNA after a 4.5-transfection period was analyzed using flow cytometry. As shown in Figure 5-7, there was no difference of cellular associated DNA among cells among the DC-Chol formulations or the QS3 series ($p>0.05$). Although cells transfected with QS3 liposome series had about two-fold greater cellular fluorescence than those treated by DC-Chol liposome series, the transgene expression were not necessarily greater for all formulations (Figure 5-2).

Structures of Complexes

The structures of complexes of DNA/liposomes were heterogeneous. The photomicrographs of major populations are shown in Figure 5-8 and 5-9. In the DC-Chol series, the micrographs showed liposomes coating the plasmids. The size of the aggregate on the micrograph was larger than 2 μm (Figure 5-8). However, the shape of the aggregate was not symmetric or ball-shaped. The narrow part of complex was less than 0.5 μm (Figure 5-8). It seemed that DC-Chol and DC-Chol/DOPE liposomes collapsed and coated the DNA (Figure 5-8 A, B) to form a spaghetti structure. The data are in agreement with Huang and coworker's results obtained by freeze-fracture electron

microscopy (Sternberg et al. 1994). However, the clumps of DC-Chol/DOPC liposomes were clearly seen coating the DNA (Figure 5-8C). This difference might result from the difference in stability of liposomes in the presence of DNA. The major structures of complexes of DNA/QS3 liposome series were crystal-like structures (Figure 5-9). DNA was condensed with liposomes and formed a multi-layer structure. No significant structure differences were found among the liposome formulations of QS3 series.

Discussion

In the present studies, we report a method of synthesis for a disulfide cationic lipid containing a quaternary amine head group. It was suggested that lipids with a quaternary amine group usually demonstrate greater transfection activity for *in vivo* studies (Song et al. 1997). The common method, in which a quaternary amine group is generated by refluxing a tertiary amine lipid with methyl iodide (Felgner et al. 1987), is not suitable for a disulfide-containing lipid because of its susceptibility. In our method, we used dimethyl sulfate, which can quaternarize the tertiary amine group of disulfide lipids under milder conditions so that the disulfide linker keeps intact. This method may be applied to prepare other sensitive cationic lipids.

The helper functions of DOPE *in vitro* transfection are well known but not fully understood. The data generated in SKnSH cells is consistent with the current literature, but the information from the CHO tissue culture studies indicates that other factors may also have importance. The helper function of DOPC has rarely been reported (Farhood et al. 1995, Felgner et al. 1995, Gao and Huang 1991, Lee et al. 1996, Tang and Hughes 1998, Xu and Szoka 1996, Yang and Huang 1998). Although the mechanism of cationic liposome-mediated plasmid delivery is not fully understood, it is generally believed that

DNA/liposome complexes enter cells via endocytosis followed by release of DNA to cytosol (Xu and Szoka 1996, Zhou and Huang 1994). The major barriers involved in the plasmid DNA delivery process *in vitro* generally include the following steps (Zabner et al. 1995): 1. Uptake of the complex or DNA into cell lines; 2. Escape of DNA or complexes from the endosomes (if uptake by endocytosis); 3. Dissociation of DNA from liposomes; 4. Entry of DNA into nucleus; and 5. DNA transcription. A strategy to overcome any of five barriers should increase transgene expression, and formulations, which overcome the major barriers, will result in greater transgene expression. Helper lipids could possibly assist in any of the above steps, and may also serve as important structural components allowing for liposomes to form. Some cationic lipids alone would only form aggregates and be unsuitable for transfection studies. It is clear that particular lipids may also have distinct influences on cellular function and may assist or deter DNA processing. DOPE is a phospholipid, which has a relative small primary amine headgroup compared to its lipophilic domain and exhibits a high tendency to form inverted hexagonal structures in the correct environment, whereas DOPC has a quaternary amine headgroup and has no activity in forming reverse hexagonal structures. Reverse hexagonal lipid phases are proposed intermediates in membrane fusion (Koltover et al. 1998). Therefore, DOPE instead of DOPC is expected to help to overcome the second barrier by assisting in the destabilization of the endosomal membrane (Koltover et al. 1998, Xu and Szoka 1996, Zhou and Huang 1994). According to our data, liposomes composed of DOPC demonstrated greater transfection in CHO cells than liposomes with or without DOPE. The helper function of DOPC in CHO cells was also reported by Hui's group (Hui et al. 1996). Hui and coworkers rationalized that (1, 2-dioleoyl-3-

trimethylammonium-propane) DOTAP/DOPC liposomes delivered a greater amount of DNA into cells than DOTAP/DOPE liposomes, thus achieving a greater transgene expression. Our experiments were designed to address in which step DOPC plays an important role.

The particle sizes of DNA/liposome complexes in serum free media for all treatments were measured and sizes ranged from 500 nm to 4000 nm (data not shown). No correlation between the sizes of complexes and liposomes with or without helper lipids was observed. In addition, no correlation was found between transfection activities and sizes of complexes. The correlation between the complex size and transfection activity is a very controversial topic. If endocytosis is a major pathway of particle entry, particles smaller than 200 nm should be internalized easier than larger particles. Stegmann and coworkers reported lack of a correlation between complex size and transfection *in vitro* (Stegmann and Legendre 1997). Liu and coworkers also reported lack of a correlation between *in vivo* transfection and particle sizes (Song et al. 1997). However, Hui's group reported that large particles (>1 μm) were more efficient for the transfection of cultures cells (Ross and Hui 1999). The picture becomes even more clouded due to the heterogeneous nature of the final product and the fact that no one has isolated the most active species in the DNA/liposome mixture (Aronsohn and Hughes 1998). There are also difficulties in obtaining realistic measurements of the complexes in solution. When aggregates are irregularly shaped (Figure 5-8), light scattering results can not reflect the diameter of all directions since there is no existing model for the scattering patterns of irregularly shaped particles (Row 1993).

It is interesting to note that DOPE increased the toxicity of DC-Chol (Figure 5-4A). The toxicity of free liposomes was also compared in CHO cells and DC-Chol/DOPE liposomes demonstrated greater toxicity than DC-Chol and DC-Chol/DOPC (data not shown). The higher toxicity of DC-Chol/DOPE might be caused by the efficient release of endosome contents and cationic lipids which may impact cellular biochemistry (Farhood et al. 1992). The difference in toxicity might contribute to the alterations in the transgene expression. Since the toxicity of DC-Chol/DOPC was lower than DC-Chol/DOPE, it is possible that more cells survived thus resulting in greater amount of expressed protein when treated by DNA/DC-Chol/DOPC. The toxicity data in QS3 or QN3 formulations were different from that of DC-Chol formulations (Figure 5-4B,C). DOPE did not increase toxicity of QS3 or QN3. A possible explanation is that QS3 or QN3 lipid has relatively low toxicity compared to DC-Chol, the release of more QS3 or QN3 lipid by DOPE did not lead to a significant enhancement of toxicity. At the high concentration, QN3 showed greater toxicity than its disulfide counterpart QS3. Since no significant difference of the toxicity was found among formulations of QS3 or QN3 (Figure 5-4 B, C), toxicity was not a factor resulting in the greater transfection activities of QS3/DOPC and QN3/DOPC in CHO cells.

Chloroquine is a base known to interfere with the endocytosis pathway by buffering the pH within endosomes and inhibiting the escape of endocytosed substances (Dean et al. 1984, Mellman et al. 1986). The transfection activities of all formulations with DOPC were dramatically reduced by chloroquine (Figure 5-5A). The transfections of liposomes with DOPE were less affected. It is possible that the propensity of DOPE to form reverse hexagonal structures offseted part of the effect of chloroquine so that the

activities of the DOPE containing liposomes were affected to a relatively less degree. Cytochalasin B (CB) interferes with the endocytosis pathway by a cessation of the movement of ruffles and pseudopodia on the surface of the cells (Wagner et al. 1971). CB (Figure 5-5B) affected transfections of all liposomes in CHO cells. The transfection activities of QS3/DOPC and QN3/DOPC were affect more by CB. The results suggested that transfection of DOPC liposomes in CHO cells followed the mechanism of the endocytosis as a possible, or major pathway for liposome-mediated DNA delivery (Hui et al. 1996, Zhou and Huang 1994). It is not clear what is the mechanism of the differences between the two cell lines studied in regards to transgene expression. Others have reported that there was a cell line specificity to particular cationic lipids and now helper lipids. Potential explanations could include differences in cellular accumulation of the particles, their stability within the cell or tissue culture media, endosomal fate, release from the endosomes, and transcripitional activity within the individual cells. At this stage we can not associate any one of the above for the discrepancy in the two cell lines but it is an interesting point of conjecture which we will continue to investigate.

The release of DNA is one of the barriers of gene delivery (Escriou et al. 1998a, Zabner et al. 1995). The interaction of DNA and liposomes can protect DNA from degradation. However, the strong complexation may also inhibit DNA from release into cytoplasm (Bhattacharya and Mandal 1998). Since all calculations were based on the amount of cationic lipids, data in Figure 5-6B clearly demonstrated incorporation of a helper lipid did not change the interaction between DNA and QS3 liposomes series. DC-Chol liposomes and DC-Chol/DOPC liposomes also showed the same results (Figure 5-6A). The closer surrounding of plasimd DNA by liposomes when DOPE is present in

formulations was also reported by Zuidam and Barenholz (Zuidam and Barenholz 1998). In order to form spaghetti like structures with DNA, cationic liposomes need to fuse with each other and form a bilayer coating the DNA strand. The fusgenic property of DOPE makes the membrane fusion more easily to occur. However, DOPE did not show this function in complexes of DNA and QS3 liposome series and the DNA release patterns were similar among the three formulations. It may be that the structure and charge of the QS3 series is the driving force in determining the final structure and the helper lipid only contributes a minor role.

Cellular associated DNA is a parameter associated with transfection. Different from Hui and co-worker's data (Hui et al. 1996), our data showed no significant difference in cellular associated DNA facilitated by the helper lipids. Therefore, the helper function of DOPC can not be explained by that it was via delivering a greater amount of DNA. Huang and Yang previously reported that DC-Chol/DOPE liposomes delivered 10 times lower amounts of DNA to BL6 cells in the presence of serum than in the absence of serum whereas the transgene expression levels were the same. Huang rationalized that some components of serum may have played a dominant role in DNA trafficking to the nucleus from the cytoplasm (Yang and Huang 1998). After complexes were endocytosed, they must escape from endosomes before enter nucleus for transcription. The fusogenic properties of DOPE may help in this early stage. However, DOPC does not have this fusion property, the maturation of endosomes and fusion events still occurs but may depend on the natural lowering of pH. The physical properties of the complexes in early endosomes may be different from those from later endosomes. The complexes that escape from early or later endosomes may demonstrate different

intracellular trafficking. When the rate of escape from endosomes is not the rate-limiting step in transfection, the difference of intracellular trafficking of complexes with different helper lipids might play a key role in transgene expression. To prove this hypothesis, our future work is to identify the intracellular mechanism involved in plasmid delivery in CHO.

In summary, the helper lipid-DOPC enhanced the transfection activities of DC-Chol, QS3, and QN3 liposomes in CHO cells. The helper function was cell dependent. The intracellular mechanism may play a key role in transgene expression.

CHAPTER 6
CONCLUSIONS AND FUTURE PROSPECTS

Gene therapy provides a paradigm for the treatment of human diseases. An ideal gene delivery system should be specifically targeting, biodegradable, non-toxic, non-immunogenic, and stable for storage. Cationic lipids are widely investigated non-viral vectors. Cationic lipids can be designed to be targeting, biodegradable, non-immunogenic, and stable for storage. However, toxicity and low efficiency are two major barriers limiting the application of these vectors in clinical trials. In past two decades, hundreds of cationic lipids were synthesized attempting to improve on these functions. The synthesis strategy for most of these lipids was based on randomly designed. To thoroughly understand the *in vitro* and *in vivo* mechanism of cationic liposome-mediated plasmid DNA delivery is a prerequisite to optimize the delivery system.

The purpose of this dissertation was to use biodegradable cationic lipids to test following hypotheses: 1) cationic liposomes containing disulfide linker are stable in a non-reductive environment and able to release more DNA than their non-disulfide analogs in the presence of reductive substances; 2) disulfide cationic liposomes have greater transfection activity than their non-disulfide analogs; 3) disulfide liposomes have less cytotoxicity than their non-disulfide analogs; 4) single-tailed cationic lipid with a suitable head group functions in plasmid DNA delivery, and introduction of an ester bond

into single tail decreases toxicity; 5)DOPC may have superior helper function than DOPE in certain formulations in certain cell lines.

The first hypothesis was supported by results of DNA releasing experiments using reductive substance dithiothreitol, cell lysates, and glutathione. Complexes of disulfide or non-disulfide cationic liposomes/DNA did not release DNA after 16-20 hour-incubation at 37 °C. However, disulfide liposomes partially released complexed DNA in the presence of reductive substances. The data suggested cationic liposomes/DNA are fairly stable. The release of DNA for transcription may be a rate-limiting step for plasmid DNA delivery. The release of plasmid DNA by reductive substance from disulfide liposomes/DNA complexes may increase amount of DNA for transcription in cells.

The second hypothesis was supported by plasmid DNA delivery in COS 1, CHO, SKnSH, and primary neuron cells using DOGSDSO or CHDTAEA liposomes. Disulfide liposomes demonstrated 2-50 times greater transfection activity than their non-disulfide analogs at the optimal ratio of each lipid. Primary neuron cells are a type of cells most difficult to be transfected since this cell line is post-mitotic. DOGSDSO had very high activity in this cell line compared to commercial available DOTAP lipid. Whether DOGSDSO liposomes bring more DNA into nucleus for transcription or activate cells to dividing status to increase transcription is an interesting issue, which is worthy to investigate in the future.

The third hypothesis was partially supported by toxicity data of CHDTAEA liposomes not by DOGSDSO. DOGSDSO has a stronger disulfide linker than CHDTAEA so that CHDTAEA is easier to be reduced than DOGSDSO. The electron-

withdrawing properties of β-carboxyl groups of disulfide linker weaken the disulfide linker in CHDTAEA and make it easier to be broken, thus decreasing toxicity. The data suggested modification of disulfide linker is also an important strategy to design biodegradable cationic lipid in future work.

The fourth hypothesis was based on the published results that neither headgroup nor tail region is the determinant of the transfection activity of cationic lipids. OLON was synthesized to test the hypothesis- with a suitable head group, single-tailed cationic lipids may function in gene delivery. To decrease toxicity, an ester bond was introduced into the tail group of single-tailed cationic lipid-LHON. Both single-tailed cationic lipids OLON and LHON demonstrated greater transfection activity than double-tailed DOTAP. The result that OLON had greater toxicity than doubled-tailed cationic lipid DOTAP was expected since single tail of OLON easily inserts into cell membrane and causes membrane rupture. LHON has a biodegradable ester bond in the backbone and demonstrated less toxicity than DOTAP. The results suggested a balance between head group and tail is needed to achieve high transfection activity of cationic lipids. The transfection activity of disulfide lipids CHDTAEA or DOGSDSO may be further improved by designing new tail or head group.

The fifth hypothesis was based on the helper function of neutral lipid DOPC on pDNA transfection using quaternary amino disulfide lipids QS3 and its non-disulfide QN3 in CHO cells. Neutral helper lipid DOPE has been reported to have helper function. DOPC was seldom reported to have the helper function. The hypothesis was only supported by the superior helper function of DOPC to DOPE in formulations QS3/DOPC, QN3/DOPC and only in CHO cells. In SKnSH cells, DOPE demonstrated

greater helper function than DOPC as usually reported. To understand the possible reasons, cellular associated DNA, transfection pathways (endocytosis or fusion), toxicity, release of DNA from liposomes, and structures of complexes were investigated. None of results from above experiments could clearly explain the helper function of DOPC in CHO cells. An unkown intracellular transportation mechanism was proposed, and however, was not proved. In the future, if fluorescence labeled DOPC is available, to observe the cellular pathway of DOPC formulations may help to understand helper function of DOPC.

Finally, although the *in vitro* results demonstrated CHDTAEA could decrease toxicity and increase transgene expression of plasmid DNA delivery, and DOGSDSO and CHDTAEA also showed low toxicity and achieved promising results when used for delivery of oligonucleotides to decrease blood pressure in a rat model (Tang and Phillip, unpublished data), very low efficiency was obtained when these lipids were used for DNA delivery in a rat brain model. Several commercial cationic lipids were also tested in the system, none achieved a good result. Some unknown hurdles exist to interfere with plasmid DNA delivery in the rat brain model.

Our plans are to continue to improve biodegradable cationic lipid-mediated plasmid DNA delivery system. The major efforts will be focus on explore *in vivo* plasmid DNA delivery in a rodent brain model.

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BIOGRAPHICAL SKETCH

Fuxing Tang was born on September 17, 1966, in Pin Jiang, China. Fuxing entered East China Normal University in August 1984 and obtained his bachelor of science degree in chemistry in July 1989. He taught in the Department of Chemistry of Hunan Medical University for six years. In August 1995, Fuxing enrolled in the University of Regina, Saskachewan, as a graduate student studying in the field of organic synthesis. He transferred to the Department of Pharmaceutics of the University of Florida in January 1997. He received his Ph. D. in pharmaceutical sciences in August 2000 under the supervision of Dr. Jeffrey A. Hughes. During his free time, Fuxing enjoys playing WeiQi and reading all kinds of books.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Jeffrey A. Hughes, Chair
Associate Professor of Pharmaceutics

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Gayle A. Brazeau
Associate Professor of Pharmaceutics

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Guenther Hochhaus
Associate Professor of Pharmaceutics

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Edwin M. Meyer
Associate Professor of Pharmacology and Therspeutics

This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 2000

Dean, College of Pharmacy

Dean, Graduate School